1 *IMPROVEMENT STRATEGIES IN OVINE ARTIFICIAL INSEMINATION*

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## 12 1.- Abstract.

13 Artificial insemination in ram is scarcely widespread comparing with other domestic
14 species. This has been due not only to fertility results being irregular and low but also
15 because of the difficulty in the application of enhancements such as the use of frozen-
16 thawed sperm. Although there is a lot of information on the use of different options to
17 improve these AI results (such as transcervical application, the use of thawed sperm, etc)
18 commercial programmes can be classified on two general categories: those using
19 refrigerated semen (15ºC) by superficial intracervical deposition (vaginal), and, more
20 restricted, those using thawed sperm by intrauterine deposition (laparoscopy).

21 In the present work we have summarized our viewpoint on three general research lines for
22 the improvement of AI results in sheep: semen preservation, AI procedures and semen
23 assessment. Briefly, in ram it is necessary to develop a medium term methodology of
24 sperm refrigeration (3-5 days) which would allow the distribution of sperm doses to a
25 widespread area. Nevertheless, it is also necessary to develop an intrauterine transcervical
26 AI technique which allows thawed semen to be applied by vaginal insemination. Besides,

the low predictive value of classic assessment techniques limits the ability to adjust the number of spermatozoa per dose according to its actual fertility.

## 2.- Introduction.

The enhancement of artificial insemination is a valuable tool in genetic improvement programs and ovine breeds conservation. However, artificial insemination is not so widespread in ovine as it is in other domestic species. This has been due not only to fertility results being irregular and low, but also because of the difficulty in the application of enhancements such as the use of frozen-thawed sperm. Insemination outcome is affected by many factors (intrinsic and extrinsic) related to female (handling, seasonality, genital morphology, etc), male (seasonality, sperm quality, sperm conservation, etc), farm (environmental conditions, sanitary status, handling, etc) and the technique itself (route of application, spermatozoa/dose, technician, etc) (Donovan et al., 2004; Paulenz et al., 2004; Shackell et al., 1990).

In this sense, Anel et al. (2005) with data on AI in the Churra breed (Spanish milking breed) showed a great difference between vaginal-cervical inseminations (refrigerated sperm) and laparoscopic inseminations (thawed sperm) with regard to fertility (Table 1). This difference justifies the use of laparoscopy in many cases, since it reduces the detrimental influence of some sources of variation, which had a higher impact on vaginal-cervical AI fertility. A large part of fertility variation was due to the farm factor. Good handling practices and careful management of other sources of variation might help to prevent a low fertility outcome. When preparing ewe lots for AI, we recommend that ewes older than 5 years should not be used, that the lambing-AI interval should be longer than 10 weeks and that insemination should not be carried out during high-temperature periods (July-August).

In order to analyze the problems of AI spreading in ovine, it is necessary to know which would be the most ideal conditions for application that would ensure the development of the technique under optimal conditions. That is, the insemination at any time of the year (seasonality problem), the use of insemination with a previous treatment of induction and synchronization of oestrus, which may have a negative effects on fertility. Also, the application of frozen-thawed sperm (only useful with deep intrauterine deposition) applied by vaginal method -intrauterine transcervical- (the cervix structure is an obstacle when the intrauterine transcervical route is being used).

In the present work we have summarized our viewpoint on three research lines for the improvement of AI results in sheep: ram semen preservation, ovine artificial insemination procedures and ram semen assessment.

## 3.- Ram semen preservation.

Currently, there are two methods which allow to achieve acceptable fertility results by AI in ovine: cooling (15ºC) and freezing (laparoscopic technique). Nevertheless, ovine AI fertility depends on the interaction between the sperm preservation method and the seminal application technique (vaginal, cervical or intrauterine deposition). A methodological problem is derived from this interaction, which hinders the widespread use of techniques based on frozen-thawed ram semen. Refrigeration of samples at 5ºC may provide a solution until the problem of using thawed semen by vaginal method is solved.

### 3.1.- Storage at refrigerated temperatures.

The storage of semen in a liquid state can be achieved by methods that reduce the metabolism of spermatozoa and thereby prolong their fertile life. Liquid storage of spermatozoa is carried out using temperatures low enough to depress sperm metabolism

(5ºC or 15ºC). The storage of ram spermatozoa at a low temperature significantly affects cell viability due to the detrimental effects of cold shock. These effects can be partially overcome by gradually cooling semen from room to storage temperature and by supplementing the diluents with some additives. Also, semen storage has been carried out by reversible inactivation of spermatozoa at ambient temperatures (Salamon and Maxwell, 2000).

Many diluents have been developed to make liquid storage of semen useful for AI. At present, the most useful method for intra-cervical AI (vaginal method) is the application of cooled semen at 15ºC being the fertile life of the spermatozoa 6-8 h. However, diluted semen must have a minimum shelf-life of between 2 and 4 days in order to be used in distant locations. The evolution of semen dilution technology has showed that survival of spermatozoa for extended periods is inversely related to their metabolic activity. Storage of bull spermatozoa at 5ºC reduces metabolic activity which contributes to extended survival. Nonetheless, not all changes induced by low temperatures are beneficial to spermatozoa. For instance, the intracellular concentration of Na rises due to the depressed activity of the $Na^{\pm}/K^{\pm}$ pump at 5ºC and this effect is detrimental to the survival of spermatozoa (Vishwanath and Shannon, 2000).

It was postulated that storage at ambient temperature may be superior to storage at 5ºC but this has resulted in lower fertility. Fertility declined rapidly when ram semen stored for more than 24 h was used for cervical insemination (Maxwell and Salamon, 1993). The lambing rates for semen stored for 24, 48 and 72 h were 45-50%, 25-30% and 15-20% respectively, whereas the lambing rate for fresh semen was 65-75%.

Sperm are not exposed to aerobic conditions naturally, but no special precautions are taken to maintain anaerobic conditions during the processing of semen for AI. Observations on the effect of a gaseous environment on sperm survival have been reported at low

temperatures, where removal of oxygen was beneficial for sperm survival (Batellier et al 2001). Storage of semen packaged at 5ºC in encapsulated form has been examined in the ram. Encapsulated semen shows a reduced viability and acrosome integrity respect to the uncapsulated control but there was no difference between both kinds regarding fertilization rates (Maxwell et al, 1996).

The cytoplasm of somatic cells contains several antioxidant enzyme systems. However sperm cells are devoid of most of their cytoplasm, and therefore have little protection against ROS (Foote et al., 2002). The physiological reasons for the rapid decline in fertility of spermatozoa stored at ambient temperature are presumed to be due to three factors: extracellular oxidative stress, effects of the seminal plasma and endogenous free radical production. In general, spermatozoa deteriorate as the duration of storage increase irrespective of the diluent or temperature of storage. The motility and morphological integrity of spermatozoa are negatively affected during storage and these changes affect to the transport and survival of spermatozoa in the female reproductive tract and to their fertility (Salamon and Maxwell, 2000).

Although fertility after AI can be maintained during preservation at 15ºC for several hours, prolonged preservation reduces the fertility of the spermatozoa, probably due to stress during *in vitro* storage. The physiological processes that contribute to the aging of spermatozoa upon *in vitro* storage need to be understood to improve their fertility following AI. In fact, sperm cells are characterized by an unusual capacity to generate reactive oxygen species which cause lipid peroxidation. High levels of lipid peroxidation of the plasma membrane could greatly modify membrane stability leading to cell death. Also, reactive oxygen species seems to impair sperm cell motility and chromatin integrity. The damage of chromatin compromises the viability of the embryos. In fact, an increase in

embryo mortality associated with aging of spermatozoa has been observed for several species (Salamon and Maxwell, 2000).

Adding antioxidants (superoxide dismutase –SOD-, catalase, glutathione) to the extender has improved the percentage of motile spermatozoa and acrosome integrity of spermatozoa during chilled storage of bull semen (Foote et al., 2002). A combination of SOD and catalase in a Tris-glucose-yolk diluent had the additional effect of improving the survival of ram spermatozoa stored at 5ºC. Antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and cytochrome C improved the motility and acrosome integrity of ram spermatozoa in liquid preservation, and there was a linear improvement in survival of spermatozoa with increasing doses of antioxidants (Maxwell and Stojanov, 1996). In recent studies, catalase was found to be beneficial to bull sperm stored at 5ºC in egg yolk extender but it did not increase sperm survival in milk-based diluents (Foote et al, 2002).

Success in cooled stored semen use depends on a lot of confounding factors such as temperature of storage, composition of the extender, number of spermatozoa applied on AI, etc. (Fernandez-Abella et al., 2003). A large variety of extenders combining various components (sugar, electrolytes, buffers, egg yolk, etc) has been proposed for cooling sperm (Lopez-Saez et al., 2000). Egg yolk-based extenders are known to be practical and efficient in protecting ram spermatozoa during storage before AI (Paulenz at al., 2002). However, an extender is a complex fluid, which contains components that may be beneficial or detrimental to sperm survival. In this sense, using milk diluent to preserve ram semen at 15ºC maintains the fertilizing capacity of sperm 8-10 hours of storage (Yañiz et al, 2005).

During storage in liquid state sperm cells will generally undergo sedimentation which can cause large pH-fluctuations and a local increase the concentration of metabolic products. The solid state of storage medium could to avoid spermatozoa sedimentation reducing

changes in local chemical conditions and also diminish the metabolic consumption of mobile spermatozoa. In this sense, the successful storage of ram spermatozoa appears to be related to reversible reducing of its metabolic activity prolonging their fertile life. To increase the viscosity of the medium and thus diminish sperm motility, the addition of gelatine has been assessed in several species (Yañiz et al., 2005).

The use of cooled semen has the advantage of being easy to handle, cheap and can be used for cervical insemination. However, inadequate semen preservation is still an obstacle for the extensive use of cooled semen in sheep AI programs. The rate of temperature descent from 34-37 ºC to 15 ºC or 4 ºC is very important to diminish the cold shock (Decuadro-Hansen, 2004) and a general objective will be to evaluate the cooling procedure and addition of glycerol to egg yolk (or specific substitutes, see below frozen semen chapter) based extender. Also, the effects of adding antioxidants to the extender on viability and fertility ability of spermatozoa will be analyzed. At present, the effects of different combinations of extender, storage temperature and packaging on pregnancy rates after AI with cooled/chilled stored semen could be analyzed.

### 3.2.- Frozen-thawed semen.

Sperm freezing in ram species is currently in a stagnant situation due to the scarce diffusion of this process in its field of application. Various methods with commercial use and acceptable results have been developed (Anel et al., 2003; Maxwell et al., 1995; Moses et al., 1997) but their general application is restricted to intrauterine sperm deposition by laparoscopy, because of the low fertility rates observed with thawed semen by vaginal-cervical insemination (Curry, 2000), especially in induced oestrus. Nevertheless, freezing methods can be improved, overall those related to handling of sperm, freezeability of the cells and the increase of sperm populations recovered after cryopreservation.

1 Low fertility rates of cryopreserved ram sperm are due to ultrastructural, biochemical and
2 functional changes undergone by a large sperm population, which leads to insufficient
3 movement and loss of viability of these spermatozoa in the genital tract (Salamon and
4 Maxwell, 2000). Ultrastructural changes mainly affect sperm membranes because during
5 the frozen-thawed process there is a redistribution of lipids that alters lipid-lipid and lipid-
6 protein relations which are necessary for the normal function of sperm membranes (Parks
7 and Graham, 1992).
8 One of the main components of dilution freezing extenders in ram species is egg yolk
9 because of its properties as a stabilizer and protector of the sperm membrane during
10 cryopreservation. Nevertheless, egg yolk has certain disadvantages, such as difficulty of
11 use, great variability of composition and the risk of microbiological contamination
12 (Bousseau et al.,. 1998). As a result, many studies have been carried out, mainly in bovine
13 species, on replacing egg yolk with its by-products or non-animal substances (Aires et al.,
14 2003). A good choice could be the use of pasteurized egg yolk (van Wagtendonk-de
15 Leeuw et al., 1999, bull sperm) or the use of powdered egg yolk (Marco-Jiménez et al.,
16 2004, ram sperm). Both studies reported similar results comparing with the use of
17 untreated egg yolk.
18 The active fraction of egg yolk is a low density lipoprotein (LDL) (Watson et al., 1975) but
19 the actual mechanism has not yet been elucidated. It has been suggested that egg yolk LDL
20 may stick on cellular membranes during the freezing-thawing process, avoiding the loss of
21 membrane phospholipids and increasing the tolerance to cryopreservation process (Graham
22 and Foote, 1987). Nevertheless, the role of protein and lipid components of LDL in these
23 interactions with the sperm membrane is still unknown. Some authors (Moussa et al., 2002;
24 Amirat et al., 2004) obtained good results using LDL in bull sperm. Other substances such
25 as phosphocaseinate or beta-lactoglobulin (Batellier et al., 1997 in ovine; Leboeuf et al.,

1 2003 in caprine) have been tested in refrigerated sperm but their utility in cryopreserved
2 semen has not been demostrated.
3 The use of alternative substances (pasteurized egg yolk, etc) allows standardized protocols
4 and reduces the risk of microbiological contamination. However, a better choice could be
5 the use of non-animal derived compounds. The most interesting of them may be soya
6 lecitine, which has a high content in egg yolk-like phospholipids. Other advantages, apart
7 from its vegetal origin, are its easy handling and standardization. Some authors (Aires et
8 al., 2003; Gil et al., 2003b; van Wagtendonk-de Leeuw et al., 2000) have tested soya
9 lecitine in cryopreserved extenders, obtaining contradictory results, making further studies
10 necessary. Currently, some commercial extenders, mostly used for bull sperm, include
11 soya derivates in their formulation.
12 In ovine species, glycerol is the main cryoprotectant used. Sperm survival is highly
13 affected by glycerol handling during the cryopreservation process (Anel et al., 2003). Thus,
14 the addition of glycerol in two steps at different temperatures seems to represent the best
15 balance between cytotoxicity and cryoprotection. Nevertheless, the variability of results
16 (Anel et al., 2003; Gil et al., 2003a; Salamon and Maxwell, 1995) leads to the conclusion
17 that more studies on the addition of glycerol and its relationship with dilution extenders
18 and freezing curves are required to obtain optimum AI results. Other substances (DMSO,
19 ethylene glycol, sugars, polymers, antifreeze fish proteins, etc) have been tested but they
20 seem to be inferior in comparison with results obtained using glycerol (Salamon and
21 Maxwell, 2000).
22 Also, other compounds, complementary to cryoprotective agents, can be used in order to
23 improve freezing-thawing results in ram sperm. For example, threalose improves fertility
24 rates in thawed semen applied by cervical insemination. This protective action may be due
25 to its antioxidant effects (Aisen et al., 2005). In addition, some seminal plasma proteins

have been tested because they seem to protect the sperm membrane against cold shock and this protection could be related to a decapacitating activity (Barrios et al., 2005).

With regard to the cryopreservation process, a capital objective is the achievement of the optimum cooling rate. In ram, Byrne et al. (2000) observed good fertility results with rapid cooling rates (-5ºC/min) both *in vivo* and *in vitro*. This was also observed by Kumar et al (2003), who consider optimum cooling rates between -20 and -30ºC/min. Generally, in the frozen-thawed process, membrane damage is produced if the freezing rate is between -15ºC to -60ºC and, in ram sperm, the majority of damages occur from -10ºC to -25ºC (Salamon and Maxwell, 1995). A significant improvement could be achieved with the use of multithermic gradients (Arav et al., 2002). This allows to a continuous and progressive adjustment of cooling and ice forming crystals which significantly reduces cryodamages.

The development of techniques which allow the use of thawed semen by vaginal-cervical insemination would lead to important changes such as an increase in the number of spermatozoa per dose. It is important because high sperm concentrations require special adaptations of the cryopreservation process (Gil et al., 2002).

An interesting way of improving cryopreservation is the study of sperm subpopulations which allows to select sexed or improved sperm. Ollero et al., (1998) demonstrated that the initial heterogeneity of ejaculates is reduced by the freezing process. Therefore, it would be interesting to study the special traits of cryopreservation methods applied to sexed sperm or to some sperm subpopulations that could be of commercial interest (Suh et al., 2005; Hollinshead et al., 2003; Mocé et al., 2006).

**4.- Artificial insemination procedures.**

The ideal technique for AI in any species is the intrauterine deposition of sperm (intrauterine transcervical insemination). Indeed, this technique renders higher fertility

1 results than when semen is placed in the vagina or the distal region of the cervix (King et
2 al., 2004; Paulenz et al., 2005). Deep levels of penetration in uterus in ewes (synchronized
3 oestrus) are necessary to obtain acceptable conception rates with cryopreserved semen.
4 Laparoscopy yields good results, since it places the seminal dose within the uterine horns,
5 but it is less practical that the transcervical AI. The anatomical structure of the ovine cervix
6 effectively prevents intrauterine deposition of semen with routine methods, based on the
7 use of straight catheters. The cervix is a highly complex fibrous structure, with many folds
8 obliterating the lumen from the vagina to the uterus, which adopts a sinuous form (Halbert
9 et al., 1990a; Kershaw et al., 2005). The cervical folds —from 3 to 7— are of conical
10 shape (Halbert et al., 1990a; Kaabi, 2002), define a narrow lumen (1–3 mm of diameter)
11 and are misaligned being the lumen of the second fold generally eccentric (Halbert et al.,
12 1990a; Alvarez, 2000). Cervical folds can be classified in two morphological types: ring
13 and flap, the former being more frequent in young ewes (less than 2 years old). Flap-like
14 folds are penetrated more easily in AI (Kaabi et al., 2006).
15 The complexity of the ovine cervix affects AI catheter penetration. In fact, some cervical
16 variables are related to AI penetration: cervix length, external width (Alvarez, 2000), fold
17 number (Halbert et al., 1990a), eccentricity of the lumen of the folds (Alvarez, 2000), fold
18 type (Kaabi, 2002), distance between the external uterine orifice and the eccentric fold
19 (Halbert et al., 1990a; Kaabi, 2002). The cervical lumen has been studied using classical
20 morphometric techniques (Halbert et al., 1990a), indirect studies with silicon moulds
21 (Naqvi et al., 2005), tridimensional reconstruction from histological preparations or
22 nuclear magnetic resonance (NMR) (Alvarez, 2000). In the last study, the author reported
23 that there is great between-ewe variability, being the cause of difficulties encountered in
24 designing a standard AI catheter for this species. In fact, the lowest diameter of the cervix

lumen was found in the eccentric fold, with only 1 mm (3mm for Halbert et al., 1990c), which is an important barrier for AI with conventional catheters (Figure 1).

The variations in the fertility achieved by AI in different ovine breeds (Donovan et al., 2004) may be related to differences in the morphometric characteristics of the cervix (Eppleston et al., 1994). Indeed, Kaabi el al. (2006) carried out a morphometric study in four ovine breeds (Assaf, Churra, Castellana and Merino), showing important differences on regard to length, width, number of folds and distance between folds (Table 2), which originates breed variations in the depth of catheter penetration into the cervix. Thus, the breeds yielding lower fertility after AI (Assaf and Churra) resulted in higher cervical complexity, which hampered the insertion of the AI catheter, achieving a lesser degree of cervical penetration.

All these morphological characteristics prevent the efficient penetration of conventional AI catheters through the cervix to the uterus. The design of new catheters allowing transcervical AI in ewes must take into account these findings, and benefit from them.

### 4.1.- Vaginal or intracervical deposition.

Vaginal or intracervical insemination is the most used AI technique in sheep. It renders acceptable results when using fresh or cooled (15°C) semen, but it has several drawbacks, since it depends on short-term storage and on high spermatozoa per dose (lowering the efficiency of the ram). Catheters are similar to those used in cattle but smaller, not allowing deep insemination to be performed.

It has been showed that placing the dose inside the cervix increases fertility in comparison with vaginal deposition (Kerton et al., 1984: 53% vs. 31%; Alvarez et al., 1998: 45% vs 22%). Some authors have indicated that increasing insemination depth, restricted to the distal part of the cervix, improves fertility results (Halbert et al., 1990c; Windsor et al.,

1994; Eppleston et al. 1994), provided that the cervix is not injured. Besides, Álvarez (2000), studying the insemination depth with a straight catheter and the reflux of semen back to the vagina, found an increase in fertility from vaginal deposition (type 0) to 2 cm depth (distal part of the cervix). Moreover, semen reflux also decreased with insemination depth (Table 3 and Figure 2).

Many factors can affect the fertility of vaginal AI (Windsor, 1995, Anel et al., 2005). The most important are: the type of semen (fresh, cooled or frozen), type of oestrus (natural or induced) and insemination depth (Eppleston et al., 1994). The low fertility obtained when using frozen-thawed semen in vaginal AI is due to the detrimental effect of cryopreservation on spermatozoa, which reduces sperm transport, viability and fertility, and increases embryo mortality (Salamon and Maxwell, 1995). Thus, fertility is low, reporting 10-40% (Maxwell and Hewitt, 1986; King et al., 2004); exceptionally, several authors have reported high fertility result after inseminating ewes with natural oestrous (Olesen et al., 1993: 58%; Paulenz et al., 2005: 72%). It is surprising that, considering the advantages of using cryopreserved semen and the encouraging results found in several published articles, there are currently no commercial programs applying cryopreserved semen for intracervical AI, all of them resorting to AI by laparoscopy.

**4.2.- Intrauterine deposition.**

Ovine insemination by laparoscopy (Killen and Caffery, 1982) is the only methodology that ensures intrauterine application of semen without reduction in fertility rates. Thawed semen is always applied by laparoscopy in all AI commercial programs in ram. Laparoscopy has multiple advantages such as high fertility results: 50-80% pregnant ewes (Anel et al., 2003; Gourley et al., 1990; Maxwell et al., 1983), low number of spermatozoa per dose, 20-50x$10^6$ spzs/dose, compared with cervical insemination (Davis et al., 1984;

Maxwell, 1986), wide time margin for insemination compared with cervical insemination, 48/72h after sponge removal (Anel et al., 1992; Maxwell, 1986) and less dependence on extrinsic factors with regard to cervical insemination (Anel et al., 2005).

Moreover laparoscopy allows ovary observation (Figure 3), thus the insemination of the ipsilateral ovary is facilitated (fewer spermatozoa are needed). Laparoscopy can also be simultaneously used to diagnose the reproductive tract, thus allowing those ewes with reproductive problems (underdeveloped or malformed genitalia; ovarian or uterine adhesions, ovarian pathologies, lack of response to oestrus induction, early gestations, etc) to be removed from the insemination lot. These problems would go unnoticed when performing vaginal-cervical insemination consequently impairing fertility results (Anel et al., 2005).

At field level, laparoscopy AI have some problems related to its complexity, high cost (in comparison with animal value), the need for trained technicians and other problems related to animal welfare. Thus, laparoscopy has transitory use until problems related to the transcervical approach are solved.

The incorporation of new technologies in assisted reproduction in ram species is a reduced field but more adequate for laparoscopy insemination. Thus, poor quality sperm samples (from high genetic value animals), epididymal sperm (Kaabi et al., 2003; Garcia-Macias et al., 2005) or any problematic semen dose could be applied with high fertility guaranties when deep intrauterine or oviductal sperm deposition are used (Jabbour et al., 1991). Also, the use of sexed semen could be included in this methodology because sorting and processing these samples affects sperm freezability (labile sperm populations). The low performance and low number of recovered spermatozoa of semen sorting justify the use of laparoscopy insemination in order to improve the profitability with good fertility results

(Evans et al., 2004). Moreover, laparoscopy IA increased efficiency in the production of suitable ovine zygotes in IVF programs (Ehling et al., 2003).

Intrauterine sperm deposition by transcervical method would be the ideal methodology for ram AI. In spite of the number of experiments that have been carried out, there is currently no protocol with acceptable fertility rates. The aim of this methodology is to reach uterus without cervix traumas in a minimum amount of time. There are several methods for crossing the cervix in ewe:

-Physical methods: attaching a haemostatic forceps to the external cervical os and retracting the cervix to align the cervical os with the opening of the rings and decrease obstructions to the uterine lumen (method by Andersen et al., 1973; method by Guelph, Halbert et al., 1990b and 1990c). These methods are traumatic (Campbell et al., 1996) and their commercial use is limited.

-Chemical methods: dilating the cervix with different hormones (oxytocin, $PGE_2$). The value of oxytocin as cervical dilator and its effect on fertility are controversial. Pharmacological doses of oxytocin have been shown to relax the cervix and improve cervical penetration but cervical traumas are still evident (Khalifa et al., 1992; Sayre and Lewis, 1997). Stellflug et al., (2001) observed a negative effect in fertility per se. Kaabi (2002) did not find an increase in cervical penetration when 200 IU of oxytocin were injected and also showed a reduction in fertility results (38.24% for the control group and 16.92% for the oxytocin group respectively). King et al. (2004) concluded that oxytocin administration as a cervical dilator cause a decrease in lambing rates (42% vs. 10%, control group vs. oxytocin group).

-Mechanical methods: due to the difficulty in crossing the cervix by physical or hormonal methods, the design of equipment which adapts to the anatomy of the cervix could be the best way of achieving transcervical AI (Wulster-Radcliffe and Lewis, 2002).

Several transcervical AI catheters have been used for passage through the cervix: pipette with eccentric tip (Buckrell et al., 1992; Halbert et al., 1990b), pipette with bent tip (Buckrell et al., 1994; Kaabi et al., 2000; Naqvi et al., 1998), helicoidal pipette (Eppleston et al., 1994), semiflexible catheter (Wulster-Radcliffe et al., 2002), etc.

Based on previous reports on the minimum diameter of cervical lumen (1 mm) and the high individual variation, Kaabi (2002) designed various catheters for transcervical AI (Figure 4). Three major characteristics of these catheters are: minimum diameter, eccentric tip (which allows the passage through eccentric rings), and rigidity (more effective in progression but also more traumatic in comparison with flexible catheters). Modified catheters achieve uterus in a high proportion of attemps (60-85%) but fertility results are lower (from 10 to 40%) when compared with uterine deposition by laparoscopy (50-70%) (Buckrell et al., 1993; Windsor et al., 1994, Wulster-Radcliffe et al., 2004). Kaabi (2002) observed that this fertility reduction is related to zone and penetration degree in the cervix. Fertility increased significantly up to 3 cm in depth, but deeper cervical inseminations or transcervical intrauterine result in a decreased fertility rates (Table 4).

In order to explain this decrease in fertility, Kaabi (2002) carried out cervical manipulations (identical to those performed in transcervical insemination) during intrauterine insemination by laparoscopy. As we can see in Table 5, deep cervical manipulations reduced fertility rates.

In this sense, Wulster-Radcliffe et al., (2002 and 2004) obtained inferior fertility results with a semiflexible catheter in transcervical insemination (4.0%) in comparison with laparoscopy insemination (41.0%). Their hypothesis is that cervical manipulations associated with catheter could activate pathways that interrupt pregnancy between days 3 and 14. In conclusion, they checked the elimination of any visual evidence of trauma and

they also observed that the procedure does not seem to affect sperm transport or embryo survival until day 3.

Transcervical insemination with catheters could induce subtle damages in sensitive zones of the cervix (delayed effects in embryonic implantation), and also unleash hormonal imbalance in postovulatory period (Raynal and Hodeau, 2004). These authors showed an increase in oxytocin as response to adrenergic stimuli (induced by vaginal speculum) and this increase could be related to reduced fertility.

Summarizing, mechanical aspects of transcervical AI seem to be solved but the fertility results for this methodology are still reduced. Further studies are necessary for the improvement of this technique.

## 5.- Assessment of ram sperm.

Fertility is a high complex biological function which depends on various cellular properties such as spermatozoa movement, recognition signals among cells, cell membrane properties, chromatin status, etc. Therefore, fertility assessment must be a compound process including evaluation of different traits of sperm function.

In the case of ram, due to specific problems in AI, the development of sperm assessment techniques predictive to field fertility constitutes an important aim in the progress of assisted reproduction. It has been demonstrated that conventional techniques are not able to accurately and repeatedly estimate the fertility of a semen sample (Correa et al., 1997). The development of techniques that pursue to evaluate the functional status of sperm organelles (acrosomes, mitochondria) or the integrity of many cellular components (membranes, chromatin) have been gaining importance during the last decades (Martinez-Pastor et al., 2004). Furthermore, the development of these techniques would enable the accurate daily evaluation of sperm batches. Besides, they could be used for the evaluation and enhancement of preservation protocols. The use of techniques with a high predictive value

for fertility would result in improvements in AI, offering the possibility of intravaginal deposition when applying thawed semen.

Sperm fertility traits can be classified into compensable -meaning that the deficiency can be removed by increasing sperm dose- and uncompensable- when the deficiency cannot be eliminated by increasing sperm dose alone. Although the compensable aspects of semen quality address the ability of inseminated sperm to access the oocyte, the uncompensable aspects address the quality or competence of the fertilizing sperm to continue or sustain the fertilization process and hence the resulting embryo (Evenson, 1999). It would be interesting to detect those rams without compensable sperm defects because the result would be high availability of sperm doses in these males.

**5.1.- Basic techniques.**

Currently, sperm assessment in AI centres is based on conventional techniques such as external aspect (volume and colour), subjective motility (wave move and individual motility) and sperm concentration (mainly using a spectrophotometer). These are practical and fast assays and require simple equipment that effectively detects semen samples of poor quality. These assessments could be enough when cervical insemination is applied because there is a high number of spermatozoa per dose, so it makes up for compensable defects. Nevertheless, better estimation of sperm quality is required when thawed semen is used. Furthermore, in specific situations (subfertility problems, accurate sperm evaluation of males, etc) these techniques are insufficient due to their high subjectivity, low sensitivity and poor correlation with field fertility. Also, these assays are based on the detection of compensable traits and do not include uncompensable ones.

**5.2.- New trends in sperm assessment.**

These assays have many advantages such as high sensitivity, automation and objectivity and some of them are well-correlated with field fertility. One of their drawbacks is the

1 complex and expensive equipment that usually required. We have summarized a brief
2 description of some of the most important techniques bellow:
3 Sperm motility automated assessment. Motility is commonly believed to be one of the
4 most important characteristics associated with the fertilizing ability of sperm. It is an
5 expression of viability and structural integrity of the spermatozoon. The assessment of
6 subjective motility has a reduced fertility predictive value due to the high variability
7 observed between individuals. Thus, variations of 30 to 60% have been reported in the
8 estimation of motility in the same ejaculate. Because of this, emphasis has been placed on
9 the development of objective methods to assess semen motility parameters. Some studies
10 carried out in humans and rats showed that velocity parameters have good correlation with
11 fertility in vitro or in vivo results (Verstegen et al., 2002).
12 The accuracy and precision of CASA systems have allowed to the detection of subtle
13 changes in sperm motion and therefore, improved discrimination among treatments in
14 laboratory studies of new extenders, cryoprotectants and other processes (Amann and Katz,
15 2004).
16 Besides, this technique has allowed to distinguish different sperm subpopulation within
17 sperm samples (Martinez-Pastor et al., 2005b; Martinez-Pastor et al., 2005c). The study of
18 subpopulations has recently been considered as an important tool, and many authors have
19 found that susceptibility to capacitation and fertilizing ability varies depending on the
20 subpopulation studied (Harrison, 1996; Olds-Clarke and Sego, 1992; Williams and Ford,
21 2001).
22 Kinematic aspects of ram spermatozoa were defined by Mortimer and Maxwell, 1999.
23 More recently other authors have used this automated process in this species (Marco-
24 Jimenez et al., 2005; Martinez-Pastor et al., 2004). Nowadays, some insemination centres
25 are adopting CASA analysis in their routine for sperm assessment but, generally, only as

1 standardized method to calculate total and progressive motility. Therefore, velocity
2 parameters, which are the most correlated with fertility, are not used and the evaluation of
3 sperm subpopulations in ram has not been applied yet.
4 Membrane status and sperm viability. The assessment of subtle membrane damage and
5 sperm viability is important because semen processing methods, such as freezing and
6 thawing, results in the death of large numbers of spermatozoa. Due to the need for a certain
7 population of motile, viable, non-capacitated spermatozoa with intact acrosomes in the
8 frozen-thawed AI dose, it is crucial that these sperm parameters be tested (Kavak et al.,
9 2003). Many different probes have been developed in order to assess plasma membrane
10 integrity and acrosome status, frequently using fluorescent markers. Further advantages are
11 obtained when flow cytometry is used, by assessing larger populations of cells. In ram,
12 fluorescence microscopy has been applied to assess sperm viability using
13 carboxyfluorescein diacetate and propidium iodide (CFDA/PI) (Grasa et al., 2004; Yaniz et
14 al., 2005). Other authors used the double stain SYBR-14/PI with fluorescence microscopy
15 (Diaz et al., 2002; Gil et al., 2003a; Gil et al., 2003b; Gil et al., 2002; Paulenz et al., 2002)
16 in this species. Nevertheless, although these stains discriminate between dead and live cells
17 (and also moribund ones in the case of SYBR-14/PI, Figure 5, a), but it is necessary to
18 detect subtle damages in the sperm membrane that could affect sperm functionality.
19 Asymmetry of the membrane phospholipids occurs during the early phases of disturbed
20 membrane function, before the integrity of the plasma membrane is progressively damaged
21 (Martin et al., 1995). When the cell membrane is disturbed the phospholipid
22 phosphatidylserine is translocated from the inner to the outer leaflet of the plasma
23 membrane, thus this sign of early apoptosis can be monitored by the calcium-dependent
24 binding of Annexin-V. In human, bull and boar Annexin V (labelled with FITC stain)
25 combined with PI appears to be suitable for early subtle membrane damage (Pena et al.,

2003). In ram, the information on subtle membrane damage is scarce. Garcia-Macias et al. (2003) compared bull and ram results using Annexin V-FITC with PI. Muller et al., (1999) studied the transbilayer distribution of phospholipids after cryopreservation and concluded that the cryopreservation process does not affect this distribution in intact (PI negative) cells.

Another important point related to membrane integrity is the evaluation of acrosomal status. In ram several fluorescent probes have been used for this purpose (Paz et al., 2002; Martinez-Pastor et al., 2005a; Valcarcel et al., 1997). One of the most commonly used is the double stain PNA-FITC/PI (Figure 5, b).

Mitochondrial status has an important role in sperm functionality because of its relationship with the energetic status of the cell and sperm motility, and because it has also been related to fertility (Casey et al., 1993; Kasai et al., 2002). There is an evidence that mitochondrial injury during freezing is involved in the poor fertility of frozen semen used for cervical insemination (Windsor, 1997). Thus, it would be an important assay to develop in this species. Various fluorochromes have been used for its evaluation. Rhodamine 123/PI has been applied in ram sperm, using fluorescence microscopy (Grasa et al., 2004; Windsor, 1997; Windsor and White, 1993). JC-1 is a fluorescent dye that has been successfully used with both fluorescence microscopy and flow cytometry (Diaz et al., 2003; Garner et al., 1997; Gravance et al., 2000; Thomas et al., 1998). In ram sperm, JC-1 results have been correlated with sperm motility in frozen-thawed sperm (Martinez-Pastor et al., 2004).

Capacitation status of ram spermatozoa has been evaluated using chlortetracycline (CTC) (Gil et al., 2002; Gil et al., 2000; Mortimer and Maxwell, 1999; Paulenz et al., 2002). But this staining must be used with fluorescence microscopy so it is slow and subjective. Furthermore, it only evaluates the later stages of capacitation (Ca2+ mediated changes)

(Kavak et al., 2003). There are other stainings which can be used with flow cytometry, such as the membrane fluidity marker Merocyanine 540 (Marco-Jimenez et al., 2005).

Morphology and morphometry. Normal sperm morphology may be an indicator of the fertility potential, but subjective methods of assessment have shown considerable between-technician and between-laboratory variation. The development of computer-assisted spermatozoa (CASMA: computer assisted sperm morphometry analysis) has allowed to reduce the components of variation in the evaluation of sperm morphology and to reveal subtle differences between individuals which cannot be detected using subjective methods (Figure 5, c). It is noteworthy that spermatozoa head shape may be related to sperm chromatin organization. Therefore, potential problems in DNA may result in subtle changes in sperm head shape that could be detectable with CASMA. Besides, sperm subpopulation can be defined according to the morphometry of sperm heads, and the distribution of these subpopulations may be related to the fertility of the sample.

CASMA has allowed to detect morphometric differences in sperm head dimensions between fertile and subfertile males in some species such as stallion (Casey et al., 1997). In ram, some studies have been carried out to develop accurate methods for employing CASMA of ram sperm heads (Gravance et al., 1998; Sancho et al., 1998), and others have analyzed the effect of cryopreservation in sperm heads (Lambrechts et al., 2000). The possible relationship between CASMA and fertility is still to be proved in this species.

Chromatin status. The nucleus is considered as the strongest indicator of semen quality and its assessment should be a priority in the spermiogram. With this aim, many fluorochrome-based techniques have been developed. The SCSA (sperm chromatin structure assay) is one of the most used ones in the last two decades because it combines the properties of acridine orange with flow cytometry which allows to analyze high numbers of cells in little time. This technique defines abnormal chromatin structure as an increased susceptibility of

1 sperm DNA to acid-induced denaturation in situ. Increased susceptibility to denaturation
2 corresponds to heterogeneity in the chromatin structure, which is associated with
3 disturbances during spermatogenesis or to subsequent chromatin damage, and which leads
4 to reduced fertility (Ballachey et al., 1987; Evenson et al., 2002). Some correlations have
5 been obtained between sperm motility, viability and capacitation status with SCSA
6 parameters in ram (Peris et al., 2004). These authors also observed that SCSA parameters
7 were higher because of cryopreservation, after incubating post-thawed spermatozoa for 20
8 hours in synthetic oviductal fluid. Other authors have obtained a good relationship between
9 SCSA parameters and individual male variation (Martinez-Pastor et al., 2004). Also, a
10 negatively correlation has been observed in ram between DNA fragmentation index
11 calculated by SCSA and progressive motility and percentage of morphologically normal
12 spermatozoa, and a positive correlation with the percentage of primary sperm defects
13 (Kasimanickam et al., 2006). Recently, SCSA has been applied in ram to compare the
14 chromatin status of samples obtained from caput, corpus and cauda epididymides, and
15 ejaculated sperm (Garcia-Macias et al., 2006). In other species (human, boar and bull)
16 there are studies in which fertility has been correlated with chromatin status detected by
17 SCSA (Evenson and Wixon, 2006) but there is a lack of information on such a relationship
18 in ram.
19 Reactive Oxygen Species (ROS). Excess production of ROS or a decreased level of
20 antioxidant enzymes affect sperm motility and viability and cause sperm defects by
21 initiating an oxidation chain reaction damaging proteins lipids and DNA (Aitken and
22 Baker, 2004; Sikka, 2004). The sperm oxidative enzymes that help counteract the effects of
23 ROS activity include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx),
24 and glutathione reductase (Sikka, 2004). The quantification of these enzymes both on
25 spermatozoa and seminal plasma, and the evaluation of ROS effects in proteins, lipids and

DNA it would be of great interest when there are subfertility problems are present or when different preservation protocols are being tested. In ram, the adverse effects of ROS on spermatozoa are more likely to be caused by direct oxidation of proteins and membrane permeabilisation than by disturbance of lipid fluidity (Christova et al., 2004). Kasimanickam et al. (2006) obtained a negative correlation in ram spermatozoa between classical semen parameters and lipid peroxidation and GPx activity. They also observed lower lipid peroxidation and GPx activity in both seminal plasma and spermatozoa in the groups of samples that they classified as satisfactory and questionable, but the SOD was lower in the unsatisfactory group. Thus, there are indications that SOD and GPx have crucial protective roles against the toxic effect of ROS in ram-lamb semen and consequently this research line must be deeply explored.

In vitro fertility analysis. IVF of homologous oocytes is one of the most informative methods for assessing sperm fertilizing ability *in vitro*. Recently, O'meara et al., 2005 observed significant differences between high and low fertility rams regarding to the percentage of oocytes cleaved obtaining a correlation between fertility *in vivo* and cleavage rate *in vitro*. They suggested that it would be a useful method for predicting field fertility of frozen-thawed ram semen. Nevertheless, it is a laborious and complex assay (high cost and high statistical complexity, etc).

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7
8

1 Table 1.- Variation factors of AI fertility in the Churra breed (Anel et al., 2005).

| Variation factor | Total AI n = 44,448 | AIV n = 17,631 | LAI n = 26,817 |
|---|---|---|---|
| Insemination technique | *** | | |
| Farm | *** | *** | *** |
| Year | *** | *** | *** |
| Season | *** | *** | *** |
| Age of ewe | *** | ** | *** |
| Lambing-AI interval | *** | *** | *** |
| Ram | *** | *** | *** |
| Technician | *** | *** | *** |
| Cumulative number of AI/ewe | N.S. | * | N.S. |

2 AIV: vaginal-cervical insemination; LAI: laparoscopic artificial insemination
3 N.S.: non significant (P≥0.05); *: significant effect (P<0.05); **: very significant effect
4 (P<0.01);***: highly significant effect (P<0.001).
5
6

1 **Table 2**.- Length, external width and number of folds of the uterine cervix in
2 several sheep breeds (Mean±SEM) (Kaabi, 2002).

| **Breed** | **Length (cm)** | **Width (cm)** | **Number of cervical folds (n)** |
|---|---|---|---|
| Assaf | 7.38 ± 0.16 [a] | 1.31 ± 0.04 [bc] | 4.63 ± 0.14 [a] |
| Merino | 7.09 ± 0.13 [ab] | 1.44 ± 0.03 [a] | 3.84 ± 0.11 [b] |
| Castellana | 6.89 ± 0.15 [b] | 1.37 ± 0.04 [ab] | 3.97 ± 0.14 [b] |
| Churra | 6.14 ± 0.15 [c] | 1.25 ± 0.04 [c] | 4.52 ± 0.13 [a] |

3 *Different superscripts within columns indicate significant differences (P<0.05).*

4

1 **Table 3**.- Fertility according to type of insemination in Churra breed (synchronized
2 oestrous, and semen cooled at 15 C) (Alvarez, 2000).

| **Type AI**[1] | **Fertility (%)**[2] | **Absolute frequency (n)** | **Relative frequency (%)** |
|---|---|---|---|
| **0** | 22.22[abc] | 18 | 0.90 |
| **1** | 28.21[a] | 677 | 32.60 |
| **2** | 31.02[ab] | 980 | 47.20 |
| **3** | 43.18[c] | 396 | 19.10 |
| **4** | 16.67[abc] | 6 | 0.30 |

3 [1]**Type 0**: vaginal deposition; **Type 1**: minimal penetration (0.5 cm depth), total reflux; **Type 2**: 0.5-1 cm depth and partial
4 reflux; **Type 3**: 1-2 cm depth and no reflux; **Type 4**: penetration to uterus and no reflux.

5 [2]Rows with different superscripts differ P<0.001.

6

7

1 **Table 4**.- Fertility variation (%) based on insemination depth -synchronized
2 oestrus, cooled semen-(Kaabi, 2002).

| AI depth | Fertility (n) |
|---|---|
| ≤ 1 cm | 48.08 (287) [a] |
| 1 cm–2 cm | 64.29 (182) [b] |
| 2 cm–3 cm | 65.12 (43) [b] |
| 3 cm–4 cm | 31.25 (208) [c] |
| > 4 cm | 29.41 (68) [c] |
| **Total** | **46.70 (788)** |

3 *Different superscripts in the same column indicate significant differences ($\chi^2$; $P<0.05$).*

4

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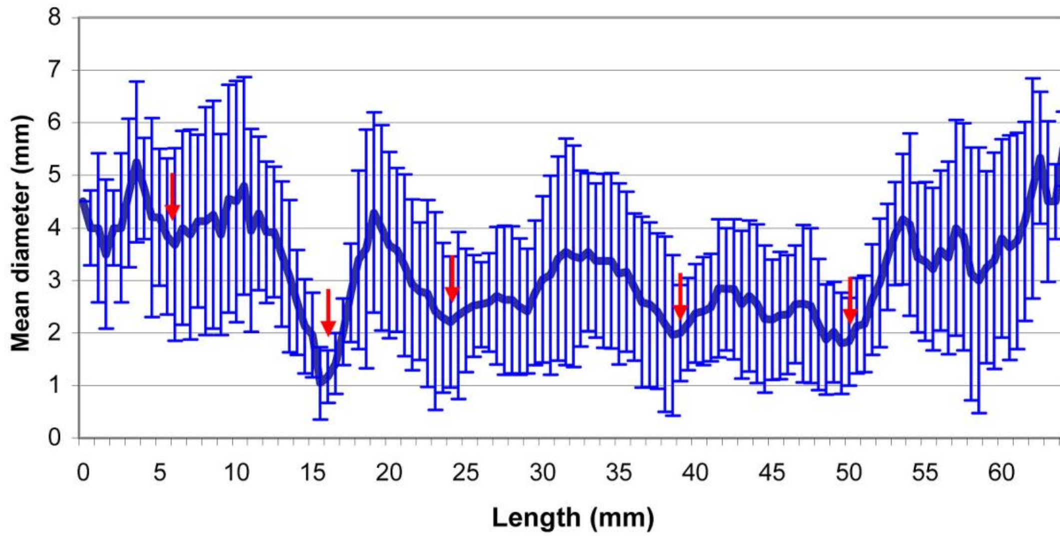
**Table 5**.- Effects of cervical manipulation on fertility results for intrauterine laparoscopy AI –synchronized oestrus, frozen-thawed semen- (Kaabi, 2002).

| Experimental group | N | Fertility (%) |
|---|---|---|
| Without cervical manipulation | 180 | 58.89 [a] |
| Cervical manipulation between 0 and 3 cm | 23 | 60.87 [a] |
| Cervical manipulation between 3 and 4 cm | 5 | 40.00 [ab] |
| Cervical manipulation beyond 6 cm (intrauterine) | 151 | 47.68 [b] |
| **Total** | **359** | **54.04** |

4 *Different superscripts in the same column indicate significant differences ($P<0.05;\chi^2$)*

5

6

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2 **Figure 1.-** Variation in the diameter of the cervical lumen (mean±SD) along the cervical

3 length, according to nuclear magnetic resonance (Alvarez, 2000). Red arrows indicate the

4 position of cervical folds.

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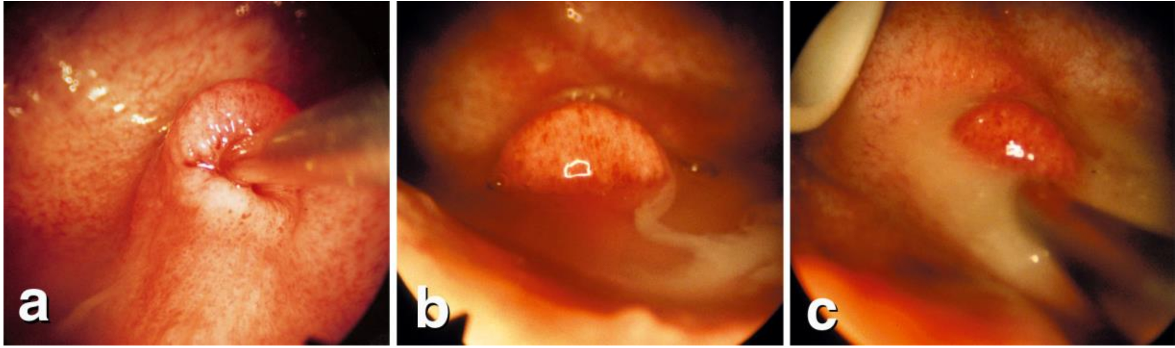
1 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3 **Figure 2**.-.Seminal reflux in vaginal-cervical insemination: (a) without reflux, (b) partial

4 reflux and (c) total reflux.

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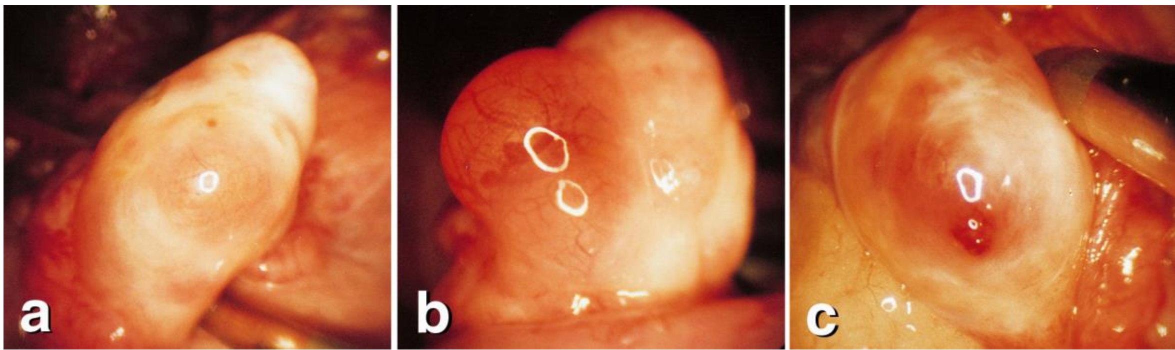


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4 **Figure 3**.- Ovarian status observed by laparoscopy during intrauterine AI: (a) follicle; (b)

5 preovulatory follicle and (c) ovulated follicle.

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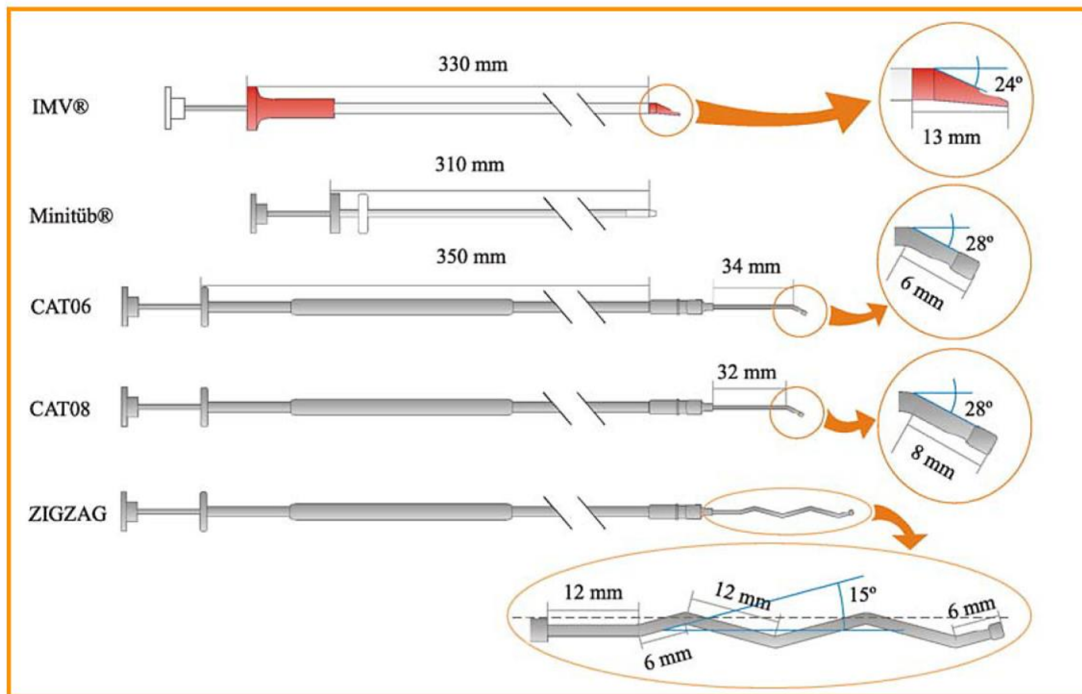


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4 **Figure 4**.- Instrumental adaptations for transcervical AI in sheep: catheters tested by Kaabi, 2002

5 (CAT06, CAT08 and ZIGZAG were designed in the University of León).

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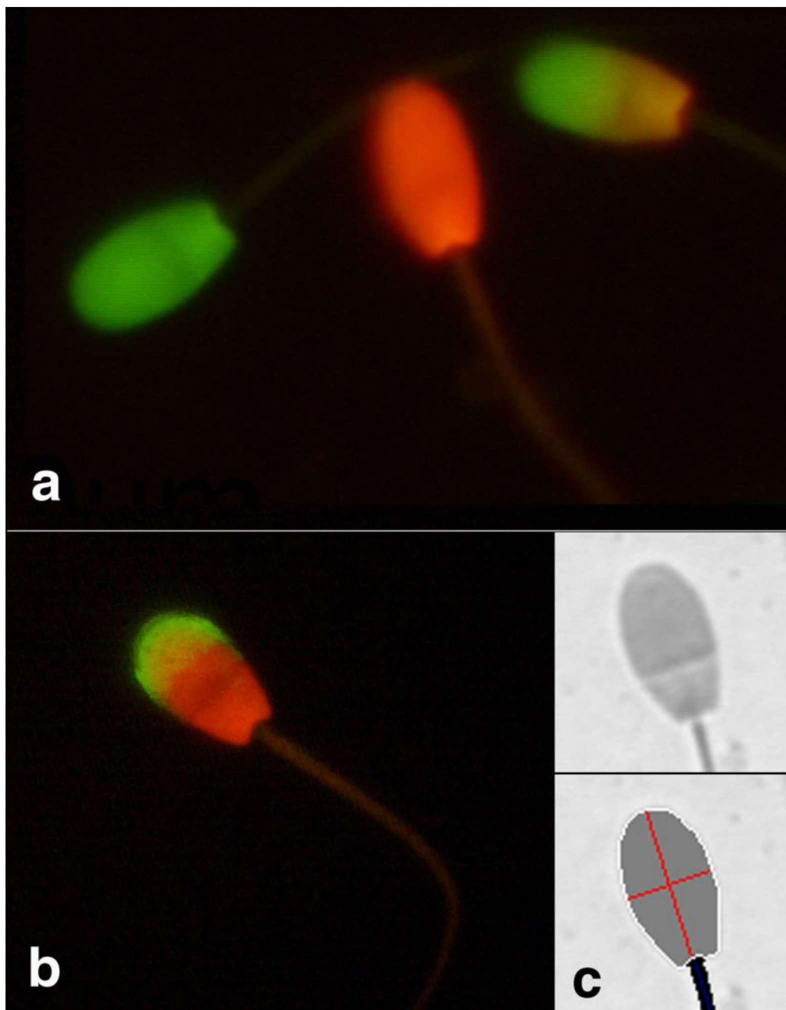


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4 **Figure 5**.- Ejaculated ram spermatoza: a) double stain SYBR-14/IP: viable (green), dead

5 (red) and moribund (green+red), b) dead with damaged acrosome by double stain PNA-

6 FITC/ PI and c) microscopic image and its mask obtained by CASMA system.

7