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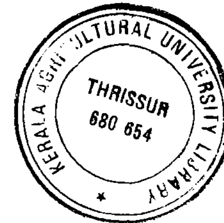
MORPHOLOGY AND VIABILITY OF BOVINE EMBRYOS FROZEN IN MEDIA CONTAINING BSA AND PROPANEDIOL

By

K. RAMACHANDRAN



THESIS

Submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Animal Reproduction
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680651
KERALA

2000

DECLARATION

I hereby declare that the thesis entitled **"MORPHOLOGY AND VIABILITY OF BOVINE EMBRYOS FROZEN IN MEDIA CONTAINING BSA AND PROPANEDIOL"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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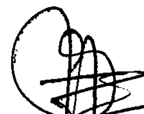
CERTIFICATE

Certified that the thesis, entitled **"MORPHOLOGY AND VIABILITY OF BOVINE EMBRYOS FROZEN IN MEDIA CONTAINING BSA AND PROPANEDIOL"** is a record of research work done independently by Dr. K. Ramachandran, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.



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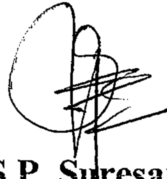

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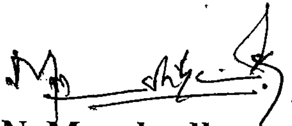

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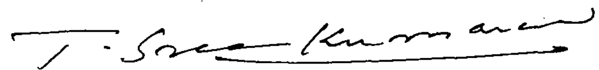

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K. RAMACHANDRAN

Dedicated to
Dr. E. Madhavan

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Introduction

INTRODUCTION

Considerable advances have been made in developing techniques to control the reproductive cycle of farm animals. Genetic improvement by intense selection and artificial insemination for economic traits have been the tool for improvement of dairy industry until recently. Further advances in reproductive techniques for control of reproduction have become necessary to exploit the full potential of these animals especially in the production area. Development of technology which enables to increase the reproductive rate of genetically superior female animals will have considerable impact on the dairy industry. One of the impediment to this has been the inability to produce more than one offspring, on an average, per year. Embryo transfer technology has thus opened new vistas in the selection process through higher intensities of selection with shorter generation intervals (Ruane, 1988). This technology can be used to cut the generation interval and to yield the same rate of genetic progress in ten years as that can be achieved in thirty years by using the conventional systems now in vogue (Arthur *et al.*, 1989).

Transfer of embryo from one female to another has been a useful tool where it is desirable to separate fetal and maternal genetic effects (Cupps, 1991). Although, embryo transfer augment conventional genetic selection, the estimated genetic progress is considered modest than that could be achieved by artificial insemination using proven sires. But the dramatic developments in the technology within a short span of time have resulted in increased demand for embryo transfer service all over the world.

Storage of mammalian embryos for extended period of time is possible only if metabolism of the embryo is effectively reduced or inhibited without causing cell injury. Cells must maintain its structural integrity all through the preservation procedure. The ability to preserve mouse embryo at low temperature (Whittingham *et al.*, 1972; Wilmut, 1972a) and the birth of calves after the transfer of blastocysts previously stored in liquid nitrogen (Wilmut and Rowson, 1973a) raised hopes in the long term preservation of embryos by deep freezing.

Several biological media have been used for the preservation and manipulation of embryos and the most commonly used one is modified phosphate buffered saline. The media used for embryo culture are also commonly employed as an advent for embryo maintenance and growth to which one or more cryoprotectants are added.

The two stages of potential danger for embryo survival during cryopreservation are the phase of initial cooling to a low temperature and the latter period of return to physiologic temperature (Mazur, 1977a).

Fetal calf serum inactivated by treatment at 56°C for 30 min is usually used in the freezing medium. Reports were seen on the use of bovine serum albumin at low levels (0.5-1 per cent), in the place of inactivated fetal calf serum, as a source of protein for embryo growth and maintenance. Polge *et al.* (1949) had reported the protective effect of propanediol during freezing. Massip *et al.* (1987) showed better results when two cryoprotectants were used for freezing.

This was used in order to minimise the osmotic injury and other specific toxic effects.

Generally, slow cooling is done to -30°C to -40°C under the conventional method, and various such protocols have been reported. Because of the toxicity at warm temperature, cryoprotectants are to be removed from the embryos. Two methods of cryoprotectant removal are employed which include a single step and multi-steps involving three to seven stages.

The present study was undertaken to compare the effect of Bovine Serum Albumin (BSA), and propanediol, on the morphology and viability of bovine embryos frozen under two different freezing and cryoprotectant removal protocols.

Review of Literature

REVIEW OF LITERATURE

Commercial exploitation of embryo transfer as a means of rapid improvement of genetic potential of livestock development, started as early as 1970 and gained momentum at a faster rate and now has become a viable biomedical technology in the livestock development programme.

2.1 Superovulation

Multiple ovulation emrbyo transfer technique (MOET) increases the reproductive potential of dam. Since the initial trials of administration of PMSG and pituitary extracts during the later stages of estrous cycle, very little modifications have been tried for superovulation in cows in the early periods (Moore and Bilton, 1977)

Although various protocols for administration of pituitary gonadotrophin have been suggested the most efficient procedure seems to be the administration of gonadotrophin during mid cycle followed by prostaglandin 48 h later to induce further regression of CL, oestrus and ovulation. Pituitary gonadotrophin such as ovine or porcine follicle stimulating hormone (FSH), because of their short half life in the cow, should be administered twice daily over approximately a four day period (Sreenan, 1983).

Hill *et al.* (1984) recommended FSH-p in 5 mg dose twice daily for six days starting on day 8 to day 13 of the estrous cycle, followed by PGF_2-α 48 to 72 h after the first FSH-p injection.

Superovulatory response in heifers with FSH-p on four different periods in the luteal phase was studied by Lindsell *et al.* (1985) and protocols starting on day 9 yielded the best results. Cloprestenol, 500 mg, was given after 72 h of the first FSH-p injection.

Schiewe *et al.* (1985) successfully administered 20 non-lactating Angus-based crossbred beef donor females, with 5 mg FSHp twice daily for five days starting from day 12 to 16 of the cycle.

The administration of prostaglandin with intervals of 48 h and 72 h after the beginning of FSH treatments has not showed any significant difference in the number of viable embryos collected per donor cow (Rodrigues and Gregory, 1986).

Zanwar and Deshpande (1988) administered 28 mg FSH in descending doses twice daily and 40 mg in constant dose of 5 mg each from day 10 to 13 with successful results.

The superovulatory response with FSH-p and ovogen was studied by Pandit (1992). In 32 mg FSH-p administered group, 55.5 per cent cows yielded embryos whereas in ovogen group (1.3 U) only 28.57 per cent yielded embryos. Nair (1992) found that 20 mg FSH with 25 mg PGF_2-α gave satisfactory

superovulatory response and better cervical dilatation; but 20 mg FSH with 15 mg PGF_2-α resulted in better embryo quality. He also found day 11 was ideal for starting the superovulatory treatment. No significant difference was seen in the superovulatory response when 32 mg FSH-p (in divided dose for four days) and 2500 IU PMSG was given (Pawshe *et al.*, 1992). Dinofertin 50 mg (30 mg in the morning and 20 mg in the evening) was given for regression of CL and induction of estrus and ovulation. The number of CL and anovulatory follicles were 10.57 ± 1.90 and 2.70 ± 0.42 respectively for FSHp group and 10.42 ± 2.37 and 4.0 ± 0.3 respectively for the PMSG group.

Bo *et al.* (1994) reported single subcutaneous injection of 400 mg NIH-FSH-p of Folltropin-V was as efficacious as that four day twice daily intramuscular injection for inducing superovulation.

Khanna *et al.* (1994) reported mean ovulation rate, average number of eggs recovered and average number of transferable embryos as 11.25 ± 2.16, 6.50 ± 1.25 and 2.75 ± 0.94 respectively in a dropping dose. Tegagne *et al.* (1994) reported that Pergovet (HMG) given at 1050 IU and Pluset (Porcine gonadotrophin) given at 1000 IU resulted in the production of more number of transferable embryos than at 1350 IU and 500 IU respectively.

Polyvinyl pyrolidone was found to be a suitable solvent for prolonging the absorption of FSH given in a single injection, providing a more practical approach to FSH administration (Yamamoto *et al.*, 1994 and Takedoni *et al.*, 1995).

Siddiqui *et al.* (1996) made a comparative study on the embryo recovery and the quality of embryos recovered, using a single subcutaneous injection on day 10 and multiple injection protocol of 49 mg Folltropin beginning on day 10 and suggested that the mean recovery and viable embryos were higher in the group which received single subcutaneous injection.

PMSG was extensively used in cattle because of its easy availability and relatively low cost but cows treated with FSH (Folltropin) was found to be significantly superior in respect of higher embryo recovery and number of transferable embryos compared to cows treated with PMSG (Folligon) (Sarma *et al.*, 1998).

2.2 Medium

The choice of media for embryo collection and temporary storage has ranged from complex culture media such as M-199 and Ham's F-10 to simplex formulations such as Dulbecco's PBS supplemented with varying amounts of blood serum. Several groups subsequently compared the progress of embryos in F-10 and PBS and found that F-10 to be superior in terms of growth, survival and hatching ability (Rajamahendran *et al.*, 1985; Smith *et al.*, 1986; Hasler *et al.*, 1987). for those operating under farm conditions the requirement may simply be for PBS containing trace amounts of antibiotics (penicillin/streptomycin) and 2% fetal calf serum (FCS) for embryo collection and PBS with 10 to 20% FCS for temporary storage prior to transfer. A series of experiments was designed by Palasz *et al.* (1995) in Canada to examine the role of serum or serum substitutes in

media used for cattle embryo transfer procedures. They had showed that biological sera or serum albumin (BSA) could be replaced in cattle embryo collection, holding, culture and freezing-thawing media by chemically defined surfactant, VF-5. It was suggested that the surfactant properties of sera or BSA were the most important components of these substances in media for seven day cattle embryos.

2.2.1 Recovery of flushing medium

The success of a flush directly related to the success of the fluid recovery. An effective flush should return 90-100 per cent of the fluid initially introduced. Some authors have reported on comparisons between the sequential uterine horn and simultaneous total uterine flush in the non-surgical recovery of embryos (Hay *et al.*, 1990), the difference in embryo recovery rate was not significant.

Recovery of 90 per cent of medium was reported by Brand *et al.* (1977) while Greve *et al.* (1977) recorded 96 per cent. Difficulty in passing catheter was reported in a few animals during flushing resulting in bleeding. Newcomb *et al.* (1978) encountered similar problems but obtained 76.9 per cent of total embryos in the first 100 ml of fluid recovered. Sreenan (1978) and Newcomb (1978) obtained better fluid recovery with larger catheters. Seidel and Seidel (1988) stated that most common error in non-surgical recovery was over inflation of the balloon leading to rupture and poor fluid recovery. Manicham *et al.* (1990) observed low flushing efficiency due to difficulty in passing catheter. Nair (1992)

reported that 20 mg FSH and 25 mg PGF_2-α was optimal for maximum recovery. Unnikrishnan (1996) reported an average of 79.5 per cent fluid recovery.

2.3 Embryo recovery

Willadsen *et al.* (1976) and Lehn Jensen and Greeve (1978) found better survival rates with blastocysts than with morulae after freezing and thawing. Sreenan (1978) observed 41 per cent embryo recovery rate on day 7 and 8 and 29 per cent on days 5 and 6. The best pregnancy rate was obtained with blastocysts of day 7 (Niemann *et al.* (1982). Using non surgical procedures, recovery was best attempted on days 6, 7 and 8 and the major problem affecting recovery rate was attributed to the operator's skill and experience (Sreenan, 1983). Donaldson (1986) observed an increase in the total number of embryos from 8.5 to 15.3 and the mean transferable embryos from 3.1 to 6.5 when the day of collection was increased from 6 to 7.5.

Subramanian *et al.* (1991) carried out non-surgical embryo recovery on day 6 of first A.I. Of the 54 embryos recovered only 31 were of transferable grade. Misra *et al.* (1992) reported that the total and viable embryo recovery was found to be better in Jersy crosses as compared to HF crosses. Day 7 embryos resulted in a higher (42 per cent) pregnancy rate than day 8 embryos(Hasler *et al.*, 1995). Megahed (1995) reported a higher pregnancy rate of 60.0 per cent of early blastocysts.

2.3.1 Evaluation and grading of embryos

The important feature of any embryo classification scheme is that it should be based on easily recognizable morphological features and should be backed with firm evidence on the pregnancy rates to be expected with each of the grades.

Morphological evaluation has been widely used to delineate embryo quality (Shea *et al.*, 1976; Elsden *et al.*, 1978; Schneider *et al.*, 1980; Shea, 1981; Wright, 1981; Madan *et al.*, 1993; Mishra and Mishra, 1998) and parameters like shape, colour, number and compactness of cells, size, number of extruded or degenerated cells were commonly used as the parameters for this purpose.

Linares and King (1980) classified embryos into three groups normal (N); in the process of degeneration (IPD) and degenerated (D).

In Ireland, a five point scale for assessing the morphology of cattle embryos before and after cryopreservation was proposed by Kennedy *et al.* (1983).

System of classifying embryos as good, fair or poor appeared to be the simplest and most reliable methods (Lindner and Wright, 1983). Gross morphologic evaluation of embryos was useful in predicting pregnancy rates for groups of embryos but is of limited value in determining survival of individual embryos (Lindner and Wright, 1983; Drost, 1986).

According to Patil *et al.* (1998) embryos were classified as transferable and non-transferable.

2.4 Embryo preservation

Chang (1947) showed that unfertilized and fertilized rabbit ova upto day 6 blastocyst stage could survive storage at low temperature. This finding had made a break through in the low temperature preservation of embryos.

Pincus (1949) stated that bovine ova kept *in vitro* at 10°C for 24 h resumed cleavage at normal rate. But the pioneer work of Smith (1952) on the effect of low temperature on the developmental stage of mammalian ova, has initiated progress in cryopreservation of embryos latter on.

Averill and Rowson (1959) made studies to determine the development of sheep ova after storing in vitro at low temperature. In the first experiment, ova were stored at 5-8°C for 6-9 hr in sheep serum and Ringers solution. No ova did develop in the Ringers solution while nine out of twelve continued development in serum. In the second experiment, lambs were born when ova were stored for 24 hr and 72 hr at 4.5-7°C in serum and in the third work, no development was seen when ova were frozen to -79°C using 12.5 per cent glycerol in serum.

Sreenan *et al.* (1970) obtained limited cleavage in seven eggs when cooled to 10°C very slowly although few embryos also showed cleavage even after rapid cooling.

Wilmut (1972b) reported that the pre blastocyst cow egg was more susceptible to chilling injury than sheep eggs. Sreenan *et al.* (1975) found that 8

celled cow eggs failed to survive cooling to 0°C whereas embryos of morulae stage survived cooling to 0°C for 15 min.

The survival and development of cow eggs in the rabbit oviduct after cooling to 0-7·5°C and storing at room temperature was studied by Trounson *et al.* (1976). They found that in PBS medium, after storing at room temperature 88 per cent of day 5 and 85 per cent of day three eggs showed normal development but in TCM 199 medium only 71 per cent of day five and 49 per cent of day three eggs showed normal development.

Using modified phosphate buffered saline supplemented with 10 per cent heat treated new born calf serum, bovine embryos were refrigerated upto 3 days without significant losses in viability (Lindner and Ellis, 1985).

Glycerol was found to be protective for embryos when stored at room temperature but not at lower temperature (Niemann, 1985). Bielanski *et al.* (1986) indicated that mouse embryos could be stored for at least 24 h with no loss of viability at refrigeration temperature. Chilling of bovine embryos could be used for routine transport over long distances for atleast 24 h (Leibo and Winninger, 1986).

The chilling sensitivity was dependent on embryo stage; the oocytes were sensitive to cooling to 0°C and under certain conditions embryos could tolerate chilling and even freezing to sub zero temperature (Pollard and Leibo, 1994).

2.4.1 Freezing

The principles of cryopreservation are believed to be the same for all living cells, the most important aspect of the process being the removal of the water from cells before they are frozen. Research in freezing techniques has involved numerous studies dealing with the type of cryoprotectant, cooling and freezing rates, seeding and plunging temperatures, thawing and methods for use in ensuring removal of cryoprotectants after thawing.

Viability of embryos after cooling is an essential pre-requisite for the successful storage at low temperature. Deep freezing and thawing were tried successfully in earlier periods with mouse and sheep embryos (Willadsen, 1977 and Whittingham *et al.*, 1979).

Slow cooling in the presence of molar concentrations of a cryoprotectant, thawing had to be slow or rapid depending on the degree of dehydration reached by the embryo (Massip *et al.*, 1987). Holding embryos constantly at low temperature was an effective method for maintaining viability during storage *in vitro* (Cupps, 1991).

2.4.2 Cryoprotectants

The injury resulting from cooling is associated with changes in the composition of the extra and intra cellular fluids during freezing and thawing. The two phases of potential danger for cell survival during cryopreservation are the phase of initial cooling to a low temperature and the latter period of returning to

physiologic temperature as suggested by Mazur (1977a). Two broad categories of cryoprotectants used are those that permeate into cells and those do not as reported by Friedler *et al.* (1988) and Niemann (1991).

2.4.2(i) Permeating agents

2.4.2(i)a Glycerol

Cryoprotective action of glycerol was demonstrated in various concentrations by Polge *et al.* (1949). Averill and Rowson (1959) used 12.5 per cent glycerol in serum for the storage of sheep ova. Goat embryos were successfully frozen using glycerol as the cryoprotectant with a slow cooling and thawing protocol (Bilton and Moore,1976). Kasai *et al.* (1981) demonstrated excellent survival rate of embryos with glycerol as the cryoprotectant in a slow cooling and thawing procedure. Rao *et al.* (1988) reported that sixty nine per cent of goat embryos after freezing in 1.5 M glycerol to have normal morphology. Miyamoto and Ishibashi (1983) while using 1 to 2 M glycerol obtained a survival rate of 70 to 90 per cent 8 cell mouse embryos.

Maria *et al.* (1985) successfully frozen 6 to 8 day horse embryos using 10 per cent glycerol as cryoprotectant. Bielanski *et al.* (1984) obtained higher survival rate of embryos in 1.5 M glycerol was used as the cryoprotectant. Similarly Slade *et al.* (1984) also obtained higher rates of equine embryos when frozen using 10 per cent glycerol. But they observed early blastocysts had better tolerated cryopreservation than later blastocysts with the same medium. Successful freezing of bovine embryos using glycerol as cryoprotectant in PBS was also

reported by Wright (1985), Takeda *et al.* (1985), Totey *et al.* (1988), Hasler *et al.* (1995) and Megahed (1995).

2.4.2(i).b Dimethyl sulphoxide (DMSO)

Dimethyl sulphoxide, like glycerol is a low molecular weight non electrolyte. It gained popularity as a cryoprotective agent after the report by Whittingham *et al.* (1972). Wilmut and Rowson (1973b) reported that eight cell cow embryos did not survive after freezing and thawing in the presence of 1.0 M DMSO whereas two of seven embryos developed when frozen in 1.5 M DMSO and warmed at 12°C/min.

Rabbit embryos better survived freezing and thawing in the medium having 1.5 M DMSO (Whittingham and Adams, 1976). Willadsen *et al.* (1976) also reported that day 5 to 8 sheep embryos survived well after being frozen slowly in 1.5 M DMSO and thawed at 12°C/min.

Willadsen *et al.* (1978) reported successful freezing of cow embryos in 1.5 M DMSO in PBS. Bilton and Moore (1979) opined that 1.5 M DMSO was equally effective as I.M. glycerol in providing protection during freezing and thawingof cattle embryos.

Kanagawa *et al.* (1979) reported successful freezing of bovine embryos in the presence of 2 M DMSO and cooling at a rate of 0.5°C/min. They did not observe any significant difference between the other cryoprotectants used. Tervit and Goold (1984) reported better survival rate of sheep embryos frozen in DMSO

or ethyl alcohol than those frozen in glycerol. Rao *et al.* (1988) observed same cryoprotective effect to 16 cell goat embryos in both glycerol and DMSO.

2.4.2.(i).c Propylene glycol

The protective effect of prophylene glycol (1,2 propanediol) was reported by Polge *et al.* (1949), Renard and Babinet (1984); and Renard *et al.* (1984) for the cryo preservation. Propylene glycol was also used successfully for cryopreservation of human ova and embryos (Lasselle *et al.*, 1985; Troth *et al.*, 1994). Suzaki *et al.* (1990) used 1,2 propanediol to freeze bovine embryos and its removal from the embryos after freezing and thawing could be carried out either with or without sucrose. Herrier *et al.* (1991) suggested that DMSO and 1-2 propanediol were appropriate cryoprotectants for IVM bovine oocytes.

Otol *et al.* (1992) reported pregnancy from embryos derived from IVM bovine oocytes and frozen using 1,2 propanediol. The fertilization rate of morphologically normal oocytes frozen in 1, 2 propanediol was reported to be higher than that of those frozen in DMSO or glycerol and 1,2 propanediol was found to be superior cryoprotectant. Otol *et al.* (1993) found that 1,2 propanediol was slightly superior to glycerol and DMSO.

2.4.2.(i).d Ethylene glycol

In *In vivo* and *in vitro* studies of goat embryos (morulae and blastocysts) it was found that ethylene glycol gave better results than using glycerol (Le Gel *et al.*, 1993). Similarly Hsu (1995) also opined better results with ethylene glycol.

2.4.2.(i).e Methanol

Methanol has colligative properties similar to other permeating agents. Leibo (1977) stated that the osmotic behaviour of methanol was similar to those observed while using DMSO or glycerol.

2.4.2.(i).f Other cryoprotectants

Dimethyl formanide and erythritol have been reported to be effective for cryopreservation of mouse and rabbit embryos. But the survival rates were found to be inferior to those obtained by DMSO or glycerol (Kasai *et al.*,1981; Miyamoto and Ishibashi, 1983).

Recent studies in short term and long term preservation of oocytes and embryos showed that certain protein known as thermal hysterists protein (THP) or antifreeze proteins (AFPs) could interact with the cell membrane and protect them during exposure to cryogenic temperature (Aray *et al.*, 1994).

2.4.2.(ii) Non permeating agents

2.4.2(ii).a Sucrose

Although completely unsuccessful as a sole cryoprotectant for deep freezing and thawing sucrose is frequently used in combination with other agents (Miyamoto and Ishibashi, 1977; Kasai *et al.*, 1981).

Massip and Van Der Zwalman (1984) found that the incorporation of 0.25 M sucrose in the freezing medium resulted in successful freezing, thawing and

transfer without dilution of the cryoprotectant. One cell and two cell bovine embryos appeared to be freezable in 2 M 1,2 propanediol and 0.5 M sucrose medium (Vincent *et al.*, 1985).

A freezing method combining 1.36 M glycerol and 0.25 M sucrose in PBS was reported by Massip *et al.* (1987).

Takahashi and Kanagawa (1988) concluded that lactose had a significant effect during freezing and thawing procedures. Moreover, embryos frozen in a glycerol-lactose mixture could be diluted directly in PBS without lactose.

Krag *et al.*, 1985; Andrade and Rodrigues, 1987; Xu *et al.*, 1988; Reichenbach and Rodrigues, 1988; Matsuoka *et al.*, 1995) reported successful freezing and transfer of embryos in medium containing sucrose.

2.4.2(ii).b Polyviyl pyrolidone (PVP)

Polyvinyl pyrolidone (PVP) a non permeating polymer was successfully used for freezing of mouse embryos (Whittingham, 1971). Because of its high toxicity, it is not recommended in embryo cryopreservation mixtures (Wilmut, 1972a; Fahy *et al.*, 1984).

2.4.3 Freezing methods

2.4.3.(i) Quick freezing

Takeda *et al.* (1984) developed a procedure where slow cooling prior to freezing was unnecessary and reported excellent survival rates after freezing.

Biery *et al.* (1986) cryopreserved mouse embryos effectively using 3 M glycerol and 0.5 M sucrose by directly pluging the straws into liquid nitrogen from ambient temperature.

A technique of spontaneous freezing of bovine blastocysts with sufficient degree of dehydration without a sophisticated apparatus was reported by Chupin (1986). Crystallisation was obtained by holding the straws vertically in the neck of liquid nitrogen container for 2 to 3 min and then pluging into liquid nitrogen and 17.3 per cent day 7 and 56.4 per cent day 8 were estimated viable after freezing.

Andrade and Rodrigues (1987) had frozen mouse embryo in two media. The straws were held vertically in the (vapour at -60°C) neck of liquid nitrogen container for 10 min and then pluged into liquid nitrogen. The survival rate after thawing was 70 per cent and 89 per cent for blastocysts in medium 1 and 73 and 100 for 8 cell embryos in medium 2.

Xu *et al.* (1988) reported a high level of success by holding the straws vertically in the neck of liquid nitrogen vapour at -54°C for 5 min and transferring to liquid nitrogen and later thawing in 0.5 M or 0.75 M sucrose solution for 12 min and culturing.

Reichenbach and Rodrigues (1988) were of the opinion that 2.5 M glycerol and 0.5 M sucrose were the best combination and it was possible for mouse embryos to survive cryopreservation by direct plunging into liquid nitrogen. There was no significant difference reported in viability between morulae and blastocysts.

The quick freezing of bovine embryos using 3 M glycerol with 0.125 M sucrose and 0.25 M sucrose did not give any survival rate (Masaokishi, 1990).

In vitro produced bovine embryos were successfully cryopreserved by ultra rapid freezing using ethylene glycol and trebelose or sucrose (Matsuoka *et al.*, 1995). They had transferred the embryos directly without dilution.

2.4.3.(ii) Vitrification

Vitrification is the solidification of liquid brought about by an extreme elevation in viscosity during cooling so that the solution is said to become a glass. This requires high concentration of cryoprotective agents, high cooling and warming rates so that both the intracellular and extracellular compartment are vitrified simultaneously.

Fahy *et al.* (1984) developed a cryoprotective medium containing DMSO and polymers which supported cryopreservation of mouse embryos by vitrification.

A mixture of 10 per cent glycerol and 20 per cent 1,2 propanediol in PBS as intracellular and 25 per cent glycerol and 25 per cent 1,2 propanediol in PBS as extracellular cryoprotective media were used successfully for the preservation of bovine embryos by vitrification (Massip *et al.*, 1987). Dilution was done in IM sucrose. The pregnancy rate after transfer of late morulae and early blastocysts was 39.1 per cent.

Vitrification solution containing 20 per cent glycerol, 20 per cent 1,2 propanediol and 1 M sucrose, was reported to be toxic to bovine demi embryos (Bielanski and Hare, 1988).

Ali and Shelton (1993) reported successful vitrification of sheep embryos. Highest survival (83.2%) *in vitro* was obtained when embryos were exposed to 30 per cent VS 11.

In vitro survival rate was significantly higher and highest developmental rate was seen for blastocyst, after vitrification (Ishimori *et al.*, 1992; Horlacher and Brem, 1994).

When embryos were pretreated in a dilute (10-20 per cent) ethylene glycol solution for 5 min, followed by short exposure to 40 per cent ethylene glycol at 20°C, the post vitrification survival rate increased to 83-84 per cent. The post thaw survival rate was reported to reach 94 per cent when men embryos were exposed to 40 per cent ethylene glycol at 25°C before vitrification (Zhu *et al.*, 1993).

According to Dinnyes *et al.* (1994) further studies were needed to improve the survival rate before bovine IVM FC morulae and blastocysts could be cryopreserved by vitrification.

After vitrification, the percentage of the survival rates at 24 h culture of porcine blastocysts, expanded blastocysts and hatched blastocysts were 41, 61 and 23 per cent respectively (Kobayashi *et al.*, 1994).

Supplementation of egg yolk in the sucrose diluent was reported to increase the post thaw viability of bovine IVM-IVF blastocysts cryopreserved by vitrification (Kuwayama *et al.*, 1994). The survival rates of morulae, blastocysts and expanded blastocysts after vitrification were 63, 97 and 90 per cent respectively (Kuwayama, 1995). Saha *et al.* (1995) reported that day 7 blastocysts survived well vitrification and direct dilution.

2.4.3.(iii) Conventional freezing

Deep freezing and transfer of embryos is well established and commonly employed in cattle. Generally, for the cow embryos, slow cooling is terminated between -30°C to -45°C before transfer to liquid nitrogen and warming is carried out in a water bath at 20°C or 37°C as suggested by Massip *et al.* (1987).

Highest survival rates of 88.6 ± 1.60 and 93.5 ± 2.6 per cent were obtained when mouse embryos were cooled at 0.5°C/min to -60°C or -70°C before plunging to liquid nitrogen (MakotoHirako, 1984).

Tervit and Goold (1984) reported that the post thaw survival of sheep embryos frozen in DMSO was better than in glycerol. The initial cooling rate was 1°C/min to -7°C, then 0.3°C/min to -35°C and to -38°C at 0.1°C/min and finally plunging into liquid nitrogen.

Slade *et al.* (1984) working on equine embryos, reported that early blastocysts tolerated cryopreservation better than larger blastocysts when frozen in medium containing 10 per cent glycerol plus 5 per cent heat treated FCS in PBS.

Cooling was initially at a rate of 4°C/min to -6°C and further to -35°C at a rate of 0.3°C/min and changing to 0.1°C/min to -38°C before plunging to liquid nitrogen.

The effect of final temperature prior to plunge in liquid nitrogen on the survival of mourine and bovine embryos was investigated by Bielanski *et al.* (1984). Embryos were cooled to -7°C at a rate of 1°C/min and further cooled to -30°C or -35°C at a rate of 0.3°C/min. Data indicated no difference in the survived rate when plunged at -30°C or -35°C in liquid nitrogen. Similarly, the post thaw survival of mouse embryos was not found to be different when the plunge temperature was -35°C or -80°C (Schmidt *et al.*, 1985).

But Leibo (1985) opined that when cooling was done initially at a rate of 1°C/min to -35°C before plunging gave better survival rate than those transferred to liquid nitrogen at -25°C and -30°C.

Takeda *et al.* (1985) reported 54 per cent pregnancy after non-surgical transfer of frozen bovine embryos. The embryos were cooled at a rate of 0.5°C/min from -5°C to -30°C before plunging to liquid nitrogen.

Maria *et al.* (1985) successfully frozen horse embryos in 10 per cent glycerol in PBS with an initial cooling of 1°C/min to -6°C. The rate was further reduced to 0.3°C/min before transferring to liquid nitrogen.

There was no significant difference reported in the pregnancy rate when morulae, early blastocysts and blastocysts were frozen in 10 per cent glycerol and 4 per cent BSA in PBS, with an initial cooling of 1°C/min to -6°C and to -35°C at

0.3°C/min and then at a rate of 0.1°C/min to -38°C before plunging into liquid nitrogen (Pettit, 1985).

Expanded blastocysts had survived better than cleaving embryos and relatively more pregnancies were established (Fehilly *et al.*, 1985). They had used an initial cooling rate of 2°C/min to -6°C and then to -80°C at a rate of 0.3°C/min transferring to liquid nitrogen.

Blakewood *et al.* (1986) showed that zona pellucida free bovine embryos could survive freezing and thawing similar to zona intact embryos from the same collection. In their experiment, the embryos were frozen in medium containing 1.5 M glycerol with initial cooling of 1°C/min to -6°C and 0.3°C/min to -32°C before transferring to liquid nitrogen.

Kim *et al.* (1992) obtained a pregnancy rate of 39.4 per cent when frozen bovine embryos were transferred. The freezing was at 0.3°C/min from -6°C to -35°C and at 0.1°C/min to -38°C before transferring to liquid nitrogen. But they had obtained only 30.3 per cent pregnancy, when freezing was carried out at 0.5°C/min from -6°C to -30°C before plunging into liquid nitrogen.

Successful results were reported by Nowshari and Holtz (1993) on transferring of fresh and frozen split goat embryos without zona pellucida. They had frozen the embryos using 0.3°C/min to -32°C before transferring to liquid nitrogen. Morulae and blastocysts with morphological appearance yielded more quality and the demiembryos derived from blastocysts were superior. Moore

(1994) opined that bovine embryo frozen at a rate of 0.5°C/min from -7°C to -34°C/min gave acceptable results.

Troth *et al.* (1994) suggested that the survival rate of human prophase T oocytes was significantly higher when frozen in 1.5 M 1,2 propanidiol and 20 per cent FBS in PBS, at a rate of 2°C/min from room temperature to -7 °C and to -30°C at 0.3°C/min before plunging to liquid nitrogen.

Megahed (1995) obtained 48.7 per cent pregnancy rate when frozen bovine embryos were transferred. He had used an intial rate of cooling of 1°C/min to -6°C and 0.3°C/min to -32°C, before transfer to liquid nitrogen.

Palasz *et al.* (1995) reported lower recovery rates of mouse and bovine embryos after freezing at a rate of 0.5°C/min frm -7°c to -35°C before plunging into liquid nitrogen in the glycerol alone group than from other three groups containing glycerol and DBS and VFS.

Hsu (1995) had successfully frozen goat embryos using 1.8 M ethylene glycol and with a cooling rate of 1°C/min from room temperature to -6.5°C and further at of 0.3°C/min to -30°C before transfer to liquid nitrogen.

2.5 Thawing

Early studies indicated that slow freezing of cattle embryos to low subzero temperature (-80°C) required slow thawing; subsequently it became evident that slow freezing of cattle embryos to relatively high subzero (-25° to -30°C) required rapid thawing (Gordon, 1996).

Bilton and Moore (1979) reported that the rate of thawing required for survival was dependent upon the rate of freezing. After freezing at 0.13°C/min, thawing at 1.2°C/min gave greater survival rates than did at 2.2°C/min, whereas embryos frozen at 0.15°C/min the faster thawing rate gave greater rate of survival. Significantly higher pregnancy rate (36.5 per cent) was reported by Elsden *et al.* (1982) when the thawing was done at 37°C than at 25°C (23.5 per cent).

Agarwal and Polge (1989a) recovered 88.94 per cent mouse embryos on thawing at 35°C. Hasler *et al.* (1995) obtained 42 per cent pregnancy rate when day 7 embryos were slowly frozen and thawed at 35°C for 10 sec whereas Hsu (1995) thawed frozen goat embryos at 20°C successfully.

2.6 Cryoprotectant removal

In the conventional multistep dilution procedure, the embryo is exposed to decreasing concentrations of the protective agent, which usually requires a microscope and a minimum of 1-2 h to be carried out under laboratory conditions. In the early to mid 1980's, an alternative to the multistep thaw procedure was described and tested by several groups, Stan Leibo and collegues in North America, Renard and Co-workers in France and massip and Associates in Belgium. In this the frozen-thawed bovine embryo was transferred from the cryoprotectant medium into one containing a hypertonic concentration of a non-permeating solute, such as sucrose.

Pregnancy was reported after thawing in water bath at 20°C and the removal of DMSO in 6 steps (Willadsen *et al.*, 1978). Elsden *et al.* (1982) also recommended the same procedure.

However, Niemann (1985) found that one step addition of a final concentration of 1.4 M glycerol resulted in equally high survival rates as with the step wise procedure. On the other hand, Wright (1985) obtained 2.6 per cent pregnancy for embryos thawed using a six step dilution of glycerol at 10 min interval. Similar observations were also reported by Takeda *et al.* (1985), Voss *et al.* (1986).

Massip and VanDer Zwalmen (1984) developed a transfer method without dilution. They mixed 1.36 M glycerol with 0.25 M sucrose in phosphate buffered saline before the direct transfer. The incorporation of sucrose in the freezing medium at a concentration of 0.25 M led to a freezing, thawing and transfer method without dilution of glycerol (Agarwal and Polge, 1989a). Embryos in 3 step dilution of cryoprotectants (glycerol and 1-2 propanediol) exhibited high survival as compared to 1 step dilution (20.23 per cent vs 6.55 per cent) (Agarwal and Polge, 1989b). But Van Waglendonk-Leeu *et al.* (1994) reported no significant difference in pregnancy rate when a single step dilution or three step dilution was done.

Hasler *et al.* (1995) successfully used a four step dilution procedure for dehydrating embryos using a decreasing concentration of glycerol and 0.3 M

sucrose. A three step dilution procedure was also reported by Voss *et al.* (1986) and Mahmoudzadeh *et al.* (1994).

2.7 Culture and viability

Early attempts at culturing early cattle embryos in Cambridge used an egg-saline medium developed by Hammond (1949(for the culture of mouse embryos. In subsequent Cambridge work on the recovery and transfer of cattle embryos, M-199 was common employed as the holding medium (Rowson et al., 1969). During the second half of the 1970's workers started using Dulbecco's PBS, either supplemented according to the modifications of Whittingham (1971) with glucose, sodium pyruvate and BSA or supplemented with FCS; the medium is now widely recognized as being particularly useful.

The short period of culture provided a rapid assessment of embryo viability (Bilton and Moore, 1979). Shea (1981) reported that better appearing embryos had a higher pregnancy rate. Oocytes matured in a defined, protein free, or a medium supplemented with 20 per cent bFF had similar rates of development to the blastocyst stage. Smith *et al.* (1986) opined F10 medium to be superior to PBS in respect to embryo growth, hatching of bovine embryos and survival during 72 h culture at 37.5°C. Dorland *et al.* (1988) reported that day 7 bovine embryos with decreasing morphological quality had lower number of nuclei, decreasing mitotic index and increase of nuclei in the GI phase.

Barone and Seidel (1986) found that supplementation of 10 per cent heat treated bovine milk resulted in a higher proportion of development of 1 cell embryos to blastocysts than supplementation with 0.3 per cent BSA.

Kane (1987) evaluated embryos by counting the number of normal blastomeres in cleavage-stage and by measuring blastocyst expansion in later stage embryos.

2.8 Luminal fluid

The significance of various bacterial constituents of cervico-vaginal and uterine fluids had been well recognised which will adversely affect the viability and fertilizing ability of sperms (Gregorie *et al.*, 1972; Gibbons and Sellwood, 1973; Hidiroglou, 1975; Prasad *et al.*, 1980 and Smith *et al.*, 1987). The importance of uterine secretions as a medium for sperm capacitation, embryo implantation and as an embryotroph to developing conceptus had led to explore its biochemical constituents.

2.8.a Alkaline and acid phosphatase (AKP, ACP)

The inorganic phosphorus content of the cervical mucus varied with the oestrus cycle, being lowest during oestrus and highest during diestrus (Moore *et al.*, 1970; Baksai, 1965).

Studies of alkaline phosphatase activity revealed marked difference between fertile and repeat breeder cows (Sinha *et al.*, 1986). In animals a score value of less than 0.5 was more conducive for fertility. Mean ACP activity of

estrual cervical mucus was significantly higher in fertile (21.0 ± 1.25 Sigma units) than infertile (5.29 ± 0.65 Sigma units) cows (Bugalia and Sharma, 1988). They also reported significant variation in AKP activity in fertile (144.09 ± 3.15 SU and in infertile (105.42 ± 3.44 Sigma Units).

Alkaline phosphatase was associated with impeding atresia and universally correlated with follicle size (Wise, 1987). Henderson and Cupps (1990) reported that high concentration of follicular phosphatase was characteristic of healthy small antral fluids.

There was no significant difference between the concentration of inorganic phosphate in cervico-vaginal mucous of cows in fertile heat and repeat breeders (Gupta, 1962; El-Nagger, 1972; and Reddy, 1973) but the concentration was significantly higher in endometritis (Gupta, 1962; Krishnaswami and Uthappa, 1984).

Significant higher values in respect of pH (6.97 ± 0.24 vs 7.61 ± 0.16) and inorganic phosphorus (1.81 ± 0.23 vs 1.36 ± 0.25 mg per 100 ml) were also reported in the cervical mucous of repeat breeding crossbred cows than in normal (Wani *et al.*, 1979).

The respective levels of Acid phosphatase (ACP) and alkaline phosphatase (AKP) in normal and repeat breeder cows in fertile and infertile estrous were 16.10 ± 2.99 vs 14.57 ± 1.57 KA units, 33.43 ± 6.10 vs 28.03 ± 4.18 KA units respectively (Salphale *et al.*, 1994). Higher alkaline phosphatase activity was reported in repeat breeder cattle during estrogenic phase (Dhable and Sharma,

2000). They had also reported significantly higher acid phosphatase level on day of estrous in repeater.

2.8.b Glucose

Results of biochemical determination indicated glycogen content of the endometrium was high during the luteal phase of the cycle and after progesterone administration in ovarioctermised cows. The level was lowest at oestrum and highest at ovarectomised conditions (Larson *et al.*, 1970).

Glycogen level or blood glucose level significantly influenced the conception rate (Goel *et al.*, 1974). Sidhu and Guraya (1985) detected very little sugar in the cervical fluid.

Marked difference in the glycogen activity between fertility and repeat breeder cows was reported by Sinha *et al.* (1986). They reported that in animals which had a score value of more than 0.5 of glycogen activity, 63.63 per cent conceived.

The glucose levels in cervical mucus and blood serum of normal cows were reported to be 1.02 ± 0.32 mg/dl and 52.23 ± 2.38 mg/dl (Sood *et al.*, 1999). They also reported that glucose levels in cervical mucus in six animals were not detectable. The mean glucose in the cervical mucus of the cows which conceived was not significantly different from the non-pregnant group (Sood *et al.*, 2000).

2.9 Progesterone profile of recipients

The progesterone concentrations in heifers that returned to estrus 18 to 20 days after mating were significantly lower than in pregnant heifers (Henricks *et al.*, 1971).

Agarwal *et al.* (1977) reported that the average progesterone concentration in non-pregnant cows was around 1.0 ng/ml during the first three days of estrous cycle, then started increasing and attained a peak value of 2.32 ± 0.44 ng/ml on day 12 and maintained till day 15 and declined abruptly.

Edqvist *et al.* (1975) observed that progesterone level varied between 1.1 and 2.8 ng/ml on day 8 of estrous cycle, and between 3.0 to 7.0 ng/ml on day 14 of estrous cycle.

At the beginning of each cycle, the progesterone level was low and increased from about day 5 to reach a level of 6.0 ng/ml between day 10 and 16 and abruptly fell to 0.5 ng/ml just prior to next estrus and the mean value was 7.24 ± 1.42 in pregnant and 0.76 ± 0.6 ng/ml in non pregnant, on day 20 (Wishart *et al.*, 1975).

The mean progestrone level after fertile and non-fertile insemination was similar from 21 days before to 13 days after insemination. In non pregnant animals the value declined while in pregnant animals the level continued to rise (Claire and Lamming, 1978).

Balakrishnan *et al.* (1986) reported that the serum progesterone level remained low during early estrus (0.38 ± 0.7 mg/ml) and gradually increased to 2.3 ± 0.84 mg/ml by day 15 of the cycle. A lower plasma progesterone level was reported in repeat breeder (Guftafsson *et al.*, 1986).

Totey *et al.* (1988) reported progesterone concentrations of 8 to 10 ng/ml in blood at day 50 in three frozen embryo recipient cows.

Pregnancy and non-pregnancy could be accurately diagnosed by blood progesterone test 20 day after AI in 91.49 and 100 per cent respectively (Spano and Rosa, 1992).

Pritchard *et al.* (1994) reported that during the expected time of maternal recognition, concentration of progestrone was higher in pregnant than in non-pregnant cows.

The average blood progesterone concentration in 84 embryo recipients immediately after embryo transfer was 3.01 and 3.2 ng/ml respectively for female which conceived and which did not. Of the receipient the progesterone concentration of less than 2.4; 2.5 to 4.9 and more than 5 ng/ml; the percentage of conception was 47.4; 10 and 40 respectively (Akelopez *et al.*, 1995).

Jayakumar (1999) reported pregnancy in 20 cow as the basis of higher serum progesterone concentration on day 20 than as day 10 after A.I. (7.006 ± 0.25 ng/ml vs 5.985 ± 0.22 ng/ml). In 17 cows which were non-pregnant the serum progesterone level was 1.576 ± 0.14 ng/ml on day 20 and 5.806 ± 0.24

ng/ml on day 10 after AI. The mean progesterone concentration on 0, 10th, 20th and 30th days were reported to be 1.98 ± 0.47, 2.43 ± 0.75, 1.97 ± 0.59 and 1.85 ± 0.78 ng/ml of serum respectively in repeat breeding cows (Dhabale *et al.*, 2000).

2.10 Pregnancy

Maximum pregnancy rate (8/12) was obtained after freezing in 1.5 M DMS=PBS and thawing at 4°C/min and by 6 step wise dilutions of the cryoprotectant (Willadsen, *et al.*, 1978) Kanagawa *et al.* (1979) had reported the birth of calf after transfer of frozen-thawed embryos.

The pregnancy rate with deep frozen and thawed embryos were reported to be lower than immediate transfer of freshly collected embryo (Schneider *et al.*, 1980). Five pregnancies each, were obtained after transferring of frozen and diluted embryos by direct transfer of frozen embryos without dilution (Massip and Van Der Zwalan, 1984). Similar finding were also reported by (Cseh *et al.*, 1994; Moore 1994 and Lange, 1995).

Takeda *et al.* (1985) reported 54 per cent pregnancy after non-surgical transfer of frozen thawed embryos. Leibo (1985) opined that cystallmic BSA could be substituted for newborn calf serum in the freezing medium and reported a higher pregnancy rate. Wright (1985) evaluated the pregnancy rate of frozen thawed embryos transferred and obtained a pregnancy rate of 28.6 per cent per embryo thawed and 33.0 per cent per embryo transferred.

Niemann *et al.* (1982) reported high pregnancy rate (62.5%) when day 7 blastocysts were transferred. Elsden *et al.* (1982) reported a pregnancy rate of 74.3 per cent for fresh and 44.5 per cent for frozen-thawed bovine embryo transferred non-surgically. Transfer of blastocysts was reported to give higher pregnancy rate than the morula (Riha *et al.*, 1987; Umasankar *et al.*, 2000).

A higher pregnancy rate was obtained when early blastocysts were frozen and transferred (Hauschultz, 1988; Megahed , 1995). The pregnancy rate for frozen embryos was approximately 10 per cent lower than that obtained for fresh embryos (Cheryl and Larry, 1988). Pregnancy rate for embryos frozen in ethylene glycol were comparable to those frozen in glycerol (Mc Intosh and Hazlogu, 1994). Overall pregnancy rate reported was 39.1 per cent for fresh and 32.5 per cent for frozen embryos (Kim *et al.*, 1992). Gustafson *et al.* (1994) reported significantly lower pregnancy rate (8 per cent) for tropisied frozen-thawed embryos transferred while for fresh embryos the rate was 39 per cent.

Cryopreserved bovine embryos yielded significantly lower pregnancy rate than freshly transferred embryos and no significant differences were found between vitrified and conventionally frozen embryos (Wurth *et al.*, 1994). Lower pregnancy rate was also reported by Riha (1994) for frozen embryos transferred.

Lee *et al.* (1993) reported pregnancy rate for fresh and frozen thawed bovine embryos as 30 and 32.5 per cent respectively. When fresh and frozen-thawed embryos were transferred, the pregnancy rate was reported to be 72 per cent and 43.9 per cent respectively (Sungur and Yurdaydin, 1994; Janowitz and

Gorlech, 1994). Transfer of fresh IVF embryos (day 7 blastocyst) resulted in 53.8 per cent pregnancy while the rate was only 42 per cent for transfer of frozen day 7 IVF blastocysts (Hasler 1992). Similar result was also reported by Massip *et al.* (1995). Their result indicated 24 per cent pregnancy rate for frozen-blastocysts as compared to 31 per cent fresh blastocysts transferred.

Materials and Methods

MATERIALS AND METHODS

Superovulation was induced in sixteen healthy crossbred cows within the range of one to four parity, kept under identical conditions of feeding and management at the Network Project on Embryo Transfer, attached to the Department of Animal Reproduction, College of Veterinary and Animal Sciences, Mannuthy. These animals were fed green fodder and concentrates as per the standard norms and requirements. Oestrous detection in these animals were done by close observation and confirmed by clinical examination.

3.1 Superovulation

All the cows were subjected to superovulation by intramuscular administration of follicle stimulating hormone (Folltropin – V, Vetrepharm, Canada (Fig. 1) at a dose of 50 mg twice daily at 12 h interval starting on day 11 (oestrum – day "O"). Prostaglandin $F_2\alpha$ (Prosolvin, Intervet, Holland) (Fig.2) 15 mg was given intramuscularly 48 h after the initiation of FSH treatment (Table 1).

3.2 Breeding of donors

Superovulated cows were inseminated at 12, 24, 36 and 48 h after oestrus detection using multiple doses of chilled semen of crossbred bulls maintained in the AI centre attached to the Department of Animal Reproduction.

3.3 Embryo collection

The donor cows were prepared for flushing as per the standard protocol (Madan *et al.*, 1993). Embryos were recovered non surgically on day 7 using two way Foleys flushing catheter. Dulbeccos phosphate buffered saline (Himedia) enriched with 0.1 per cent Bovine Serum Albumin (BSA) was used as the flushing medium. To one litre of the media, 1, 00,000 IU penicillin G sodium, 50 mg streptomycin sulphate and 1 gm dextrose were added. The medium was filtered using membrane filter (Sigma, 0.2 μ - 0.4 μ) and then autoclaved. The final pH was adjusted to 7.4 ± 0.2. Each horn was flushed with 30 to 40 ml medium at a time, till about 400 ml was used to flush each horn. The flushed medium from each horn was collected into a 500 ml sterile cylinder after filtering through an embryo filter (Minitub, Germany). Flushed medium with embryos in the filter was then transferred into sterile plastic dishes with grid (Falcon # 1012) and searched under a stereo zoom microscope (Leica, Switzerland) to locate the embryos. All the embryos located were picked up and transferred into petri dishes containing fresh holding medium (Dulbeccos phosphate buffered saline enriched with 4.0% BSA – Sigma) for grading, based on morphological characters.

3.4 Grading

All the embryos in the holding medium were graded as transferable and non transferable depending on their quality. The fertilized embryos having uniform colour, spherical shape, compact cells with intact zona pellucida were graded as transferable embryos. All embryos having morphological abnormalities

such as tight morula with oval zona, irregular and loose blastomeres, embryo with derbis, cracked zona pellucida were graded as non transferable embryos. Good embryos were further classified as morulae and blastocysts depending on the stage of development (Madan *et al*, 1993; Fig.3 and 4) for freezing.

3.5 Freezing

Within 2 h of collection, the transferable embryos were subjected to equilibration for 15 min at room temperature with 10 per cent glycerol in phosphate buffered saline. A total of 72 embryos were subjected to freezing studies. Embryos for freezing were divided into three groups, each group having 24 embryos (Table 9). Embryos in group I were frozen in phosphate buffered saline using 10 per cent glycerol as the cryoprotectant. In group II, embryos were frozen in medium having a composition of 10 per cent glycerol and 1 per cent BSA in phosphate buffered saline. Medium containing 10 per cent glycerol and 20 per cent 1, 2 propanediol in phosphate buffered saline was used for the third group.

Individual embryos transferred to each of the above freezing medium was loaded in 0.5 cc French straws (Wright, 1985). The straws containing embryos in each of the above groups were sub divided into two sets with 12 embryos each and frozen under two protocols a and b in programmable freezer (Apex Instruments, Calcutta) (Table 9).

3.5.a Freezing Protocol (a)

Initial cooling was done from the room temperature (29°C) to -6°C at a rate of 1°C/min. The straws were subjected to seeding by standard technique to avoid damage of embryos while ultra freezing and held for 5 min for equilibration of temperature. Further cooling was done to -35°C at a rate of 0.3°c/min (Fig.10). The straws were removed from the freezing chamber and immediately transferred to liquid nitrogen and stored for thirty days.

3.5.b Freezing protocol (b)

Straws with embryos were subjected to an initial cooling rate of 5°C/min from room temperature to -7°C. After seeding and holding for 5 min, further cooling was done to –30°C at a rate of 0.3°C/min, before transferring to liquid nitrogen (Fig.11). The embryos were stored for thirty days.

3.6 Thawing

After 30 days of storage in liquid nitrogen, the straws were taken and thawing was carried out by placing them in a water bath at 37°C for 20 seconds.

3.7 Cryoprotectant removal

The straws were cut and the embryos were transferred into petridishes from each sub-group and the cryoprotectants were removed using two different procedures as described by Massip *et al.* (1987) and Agarwal and Polge (1989b).

3.7(i) Four step protocol

The cryoprotectant was removed in a serial of four steps by passing the embryos (6 nos.) through phosphate buffered saline having reduced concentrations of 7.5, 5.0, 2.5 and 0 per cent at an interval of 10 min in each, and finally washed in phosphate buffered saline.

3.7(ii) One step protocol

The embryos in the group were treated with 1 M sucrose phosphate buffered saline, for 20 min at room temperature and finally washed in phosphate buffered saline.

3.8 Culture

Embryos after the final washing were examined under the microscope for the morphological changes and morphologically normal embryos were incubated in Co_2 incubator (Lab.line, USA) at 37°C under 5 per cent CO_2 tension for 24 h, using phosphate buffered saline enriched with 4 per cent BSA as the culture medium.

3.9 Viability studies

Recipient cows were selected from the University Livestock Farm and from among the cows brought to the AI centre attached to the Department of Animal Reproduction. Those animals which had normal oestrous cycle with normal breeding history were only considered for transfer of embryos. Ovulation

was confirmed by palpation of CL on the fifth day of oestrum and again on the day of transfer. The cows selected from the University Livestock Farm were treated with PG F_2 α 15 mg as per standard procedure to synchronize them. Animals selected from AI centre were those naturally synchronized with the donor. Embryos found normal after culture were transferred into the synchronized recipient cows non surgically as per the standard proctocol (Madan *et al.*, 1993) on the 8th day of the cycle after ascertaining the development of the corpus luteum.

3.10 Biochemical analysis

The luminal fluid from the recipient cows during the oestrum was collected aseptically into sterile vials and stored for biochemical analysis as described by Reddy (1973).

3.10.1 Glucose

The glucose level in the luminal fluid was estimated by O – Toluidine method (Reddy's Laboratories, 1997).

3.10.2 Acid phosphatase

Acid phosphatase was estimated by modified King's method (1959) (Reddy's Laboratories, 1997).

3.10.3 Alkaline phosphatase

Alkaline phosphatase in the luminal fluid was estimated by Kind and Kings method (1954) (Dr. Reddy's Laboratories, 1997).

3.10.4 Serum – progesterone

Blood was collected from the recipient cows on the day of heat, days 14 and 28, after oestrum. Serum was separated by centrifugation and stored at –20°C till processed for progesterone assay.

Enzyme-immunological test for the quantitative determination of progesterone was carried out by ELISA/competition using Streptavdin Technology (Morgenthaler, 1987).

Results

RESULT

The objective of the study was to compare the effect of Bovine Serum Albumin (BSA) and 1, 2 propanediol as cryoprotectants on the morphology and viability of bovine embryos frozen under two different freezing and cryoprotectant removal protocols. Sixteen crossbred cows were selected for superovulation and embryo collection. Results obtained on 1) superovulation assessed by counting the corpora lutea, 2) number of transferrable and non transferrable embryos collected, 3) long term freezing of embryos under two protocols, 4) thawing and removal of cryoprotectants under two protocols, 5) incubation and transfer to recipient cows, 6) estimation of glucose, acid phosphatase and alkaline phosphatase in the luminal fluid of the recipients and 7) hormone profile of the recipeint cows are presented in tables 2 to 22 and Fig.5 to 14

4.1 Superovulation

Superovulatory response was ascertained clinically by counting the number of corpora lutea on both ovaries (Nair, 1992). Out of the sixteen cows treated with Folltropin-V, thirteen showed good response. The right ovary showed better response with a range of 3 to 10 CL and a mean value of 5.00 ± 0.747 (Table 2). In the left ovary the number varied from 2 to 10 with a mean of 3.75 ± 0.756. The total number of CL on right ovary was eighty and on the left it was sixty.

On repeated superovulation treatment, the response was comparatively low. In the second treatment (Table 3) the number of CL ranged from 1 to 6 in the right

(mean 3.375 ± 0.596) and 2 to 7 in the left ovaries (Mean 3.00 ± 0.707) and the total number was 27 and 24 respectively. In the third trial (Table 4) the total number of CL was 14 on the right and 13 in the left and from 1 to 4 in both the ovaries (mean 2.80 ± 0.496 and 2.6 ± 0.51 respectively).

Two cows did not respond to superovulatory treatment, while cow one had multiple follicles (8 on right and 9 on left) without any evidence of ovulation. With respect to the three repeat trials in 5 animals there was a decreasing trend in the superovulatory response (Table 5 and Fig.8). The number of CL in the 1st, 2nd and 3rd trials were 73 (mean 14.6 ± 1.288), 39 (mean 7.8 ± 1.393) and 27 (mean 5.4 ± 0.927) respectively.

4.2 Flushing

Repeated non surgical flushing was done in thirteen cows. The total number of flushings were 24 (Table 2, 3, 4). Thirteen flushings were done in the first treatment, while seven flushings were carried out in the second and four in the third treatment. The fluid recovery after flushing ranged from 60 to 95 per cent. Flushings could not be carried out in three cows as there was no superovulatory response.

4.3 Embryo recovery

Embryos were recovered from twelve cows. In one cow though there was good response and 80 per cent fluid recovered, no embryos could be recovered as the flushed medium showed large quantity of mucus and debris.

It could be seen from tables 2, 3, 4, that the total number of embryos recovered were 85. Following the first treatment from 12 cows, 56 embryos (mean 3.5 ± 0.822) were recovered of which thirty were from the right and 26 from the left horn (mean 1.875 ± 0.491 and 1.625 ± 0.547 respectively). In the second treatment in six cows, 22 embryos were recovered (mean 2.75 ± 0.861), and in the third treatment in four cows, only seven embryos were recovered (mean 1.40 ± 0.40).

From the Table 6 and Fig.9, it could be seen that five cows superovulated and flushed three times repeatedly produced 34, 18 and 7 embryos in the first, second and third treatments respectively (mean 6.8 ± 1.463, 3.6 ± 1.66 and 1.4 ± 0.4 respectively).

4..4 Embryo grading

Among the 85 embryos recovered, seventy six having compact cells and intact zona pellucida were graded as transferable (89.41%), while nine were considered non transferable being unfertilised (4.70%) and morphologically abnormal (5.88%) (Table 7 and Fig.5). Out of the seventy six transferable embryos, 62 (81.58%) were morulae (Fig.3) and 14 (18.42%) early blastocysts (Fig.4) (Table 8 and Fig.6). In the first, second and third treatments the number of transferable morulae were 44, 15, 3 and blastocysts 8, 4 and 2 respectively (Table 8 and Fig.7).

4.5 Freezing

Of the 62 morulae and 14 early blastocysts, 60 morulae and 12 blastocysts were subdivided into three groups each, consisting of 20 morulae and four blastocysts and assigned to each freezing medium. Group of 12 embryos (10 morulae and 2 blastocysts) were frozen using each freezing protocol. Morphological examination after thirty days of storage in liquid nitrogen, revealed that only thirty four (26 morulae and 8 blastocysts) had normal morphology with compact cell and intact zona pellucida (Table 10).

Statistical analysis revealed significant difference between the effects of freezing media on the morphology of embryos after freezing, preservation for thirty days and the removal of cryoprotectants under two different protocols. From the table 11, it could be seen that medium II (10% glycerol + 1% BSA in PBS) (75%) was comparatively better than the other two media, 37.5% and 29.17% respectively. Significant difference was seen between media I and II and between II and III ($P<0.05$). However between the media I and III, there was no significant difference after freezing on the morphology of embryos.

There was no significant difference between the two freezing protocols and the two steps in the cryoprotectant removal on the morphology of embryos after freezing and storage (Tables 12 and 13).

4.6 Culture

Sixteen (13 morulae and 3 blastocysts) out of thirty four embryos incubated post freezing, revealed normal morphology (Table 14). Statistical analysis showed no significant difference on the morphological features between media I and II; I and III and II and III (t = 0.4767, 0.3960 and 0.2146 respectively) between the two freezing protocols and two cryoprotectant removal steps on the morphology after the culture (t = 0.4767 and 0 respectively) (Tables 15, 16, 17).

4.7 Viability studies

Sixteen embryos (13 morulae and 3 blastocysts) were selected for the transfer into the recipient animals. Fifteen cows were selected and one embryo each was transferred in 14 and two embryos in one cow. Four animals did not show any signs of oestrum at the first cycle of which one was confirmed pregnant on examination after 60 days of transfer. Two cows evinced oestrus 34 and 35 day after transfer. The third cow showed signs of oestrum after 45 days of transfer. These three animals had revealed the presence of CL (Table 22).

4.8 Biochemical studies

Nine recipient cows were selected from the animals brought to the AI centre and six from the animals maintained in the University Livestock Farm. These animals were watched for oestrum and the cervical mucus was collected for biochemical estimation.

It was seen that the mean glucose level was 128.075 ± 9.019 mg/100 ml (86.20 to 195.20 mg/100 ml). Level of acid and alkaline phosphatase ranged from 14.17 to 22.73 and 119.02 to 129.02 KA units respectively with a mean value of 18.675 ± 0.667 and 122.673 ± 0.788 KA units respectively (Table 18).

Serum progesterone of the recipient cows was estimated on days 0, 14 and 28 days after the oestrum. The value ranged from 0.01 to 4.19 ng/ml. The level ranged from 0.01 to 0.73 with a mean of 0.304 ± 0.054 ng/ml; from 2.57 to 4.12 with mean 3.25 ± 0.136 ng/ml and from 1.93 to 4.19 with a mean of 2.917 ± 0.21 ng/ml respectively for day 0, 14 and 28 days after the oestrum (Table 19 and Fig.12).

Eleven of the cows which had shown subsequent first heat after the transfer the mean progesterone levels for day 0, 14 and 28 days after the transfer were 0.357 ± 2.14; 3.053 ± 0.42 and 2.572 ± 0.627 ng/ml respectively (Table 20 and Fig.13). The mean value for the four animals which did not show any sign of heat, were 0.157 ± 0.166, 3.793 ± 0.406 and 3.67 ± 0.362 ng/ml respectively (Table 21 and Fig.14).

Table 1. Superovulatory treatment

Day of cycle	Folltropin-V (400 mg)		Prosolvin $PGF_2\alpha$
	6 AM	6 PM	7.5 mg/ml
0			
11	50 mg	50 mg	
12	50 mg	50 mg	
13	50 mg	50 mg	15 mg
14	50 mg	50 mg	
0 induced heat		AI	
1	AI	AI	
2	AI		
7	Flushing		

* Folltropin – V – Vetrepharm, Canada (FSH - P1)

** PROSOLVIN - Intervet, Holland (Luprostiol)

Table 2. Response of donors and embryo recovery to first superovulatory treatment (trial – 1)

Sl. No	Animal No	No. of corpora lutea			No. of anovulatory follicles			No. of embryos recovered			Flushing (%)	
		R	L	T	R	L	T	R	L	T	R	L
1	H 36	6	5	11	-	-	-	4	1	5	90	80
2	H 48	8	9	17	2	3	5	2	5	7	90	95
3	TM 63	6	1	7	-	2	2	1	1	2	80	80
4	A 93	7	3	10	1	1	2	3	1	4	90	90
5	150	-	-	-	-	-	-	-	-	-	-	-
6	151	5	2	7	-	-	-	3	2	5	85	85
7	152	3	5	8	1	1	2	-	2	2	75	80
8	155	4	3	7	3	2	5	-	1	1	70	70
9	327	6	2	8	-	2	2	3	-	3	90	80
10	336	8	4	12	2	1	3	7	3	10	95	95
11	448	7	10	17	2	1	3	2	8	10	95	95
12	645	-	-	-	8	9	17	-	-	-	-	-
13	719	5	6	11	2	1	3	2	2	4	80	80
14	827	5	4	9	1	2	3	-	-	-	80	80
15	914	-	-	-	-	-	-	-	-	-	-	-
16	952	10	6	16	2	3	5	3	-	3	60	80
Total		80	60	140	24	28	52	30	26	56	10.80	1090
Mean		5.0	3.75	8.75	1.5	1.75	3.25	1.875	1.625	3.50	67.5	68.125
SE ±		0.747	0.756	1.371	0.5	0.544	1.014	0.491	0.547	0.822	8.684	8.623

Table 3. Response of donors and embryo recovery for the second superovulatory treatment (trial – 2)

Sl. No.	Animal No.	No. of corpora lutea			No. of anovulatory follicles			No. of embryos recovered			Flushing (%)	
		R	L	T	R	L	T	R	L	T	R	L
1	H 36	2	2	4	-	-	-	-	1	1	80	80
2	H 48	5	7	12	-	-	-	2	5	7	80	90
3	A 93	4	3	7	-	-	-	2	1	3	90	85
4	151	1	-	1	-	-	-	-	-	-	-	-
5	336	4	3	7	-	2	2	3	2	5	90	90
6	448	6	4	10	-	-	-	2	1	3	85	90
7	719	2	3	5	-	-	-	-	-	-	90	90
8	952	3	2	5	1	1	2	2	1	3	90	80
Total		27	24	51	1	3	4	11	11	22	605	605
Mean		3.375	3.0	6.375	-	-	-	1.375	1.375	2.75	75.625	75.625
SE	±	0.596	0.707	1.224	-	-	-	0.42	0.565	0.861	10.915	10.915

Table 4. Response of donors and embryo recovery for the third superovulatory treatment (trial – 3)

Sl. No	Animal No	No. of corpora lutea			No. of anovulatory follicles			No. of embryos recovered			Flushing (%)	
		R	L	T	R	L	T	R	L	T	R	L
1	H 48	3	4	7	-	-	-	1	1	2	80	80
2	336	4	3	7	-	2	2	2	-	2	90	90
3	448	3	3	6	-	-	-	1	1	2	80	80
4	719	1	1	2	1	-	1	-	-	-	-	-
5	952	3	2	5	-	-	-	1	-	1	85	85
Total		14	13	27	1	2	3	5	2	7	335	335
Mean		2.8	2.6	5.4	-	-		1.0	0.4	1.4	67	67
SE	±	0.496	0.51	0.927				0.316	0.245	0.4	16.852	16.852

Table 5. Superovulatory response to repeat treatments (5 animals)

Sl. No.	Animal No.	Trial 1 No. of corpora lutea			Trial 2 No. of corpora lutea			Trial 3 No. of corpora lutea		
		R	L	T	R	L	T	R	L	T
1	H 48	8	9	17	5	7	12	3	4	7
2	336	8	4	12	4	3	7	4	3	7
3	448	7	10	17	6	4	10	3	3	6
4	719	5	6	11	2	3	5	1	1	2
5	952	10	6	16	3	2	5	3	2	5
Total		38	35	73	20	19	39	14	13	27
Mean		7.6	7.0	14.6	4	3.8	7.8	2.6	2.6	5.4
SE	±	0.812	1.095	1.288	0.707	0.860	1.393	0.510	0.510	0.927

Table 6. Embryo recovery in repeat treatments (5 animals)

Sl. No.	Animal No.	Trial 1 Embryos recovered			Trial 2 Embryos recovered			Trial 3 Embryos recovered		
		R	L	T	R	L	T	R	L	T
1	H 48	2	5	7	2	5	7	1	1	2
2	336	7	3	10	3	2	5	2	-	2
3	448	2	8	10	2	1	3	1	1	2
4	719	2	2	4	-	-	-	-	-	-
5	952	3	-	3	2	1	3	1	-	1
Total		16	18	34	9	9	18	5	2	7
Mean		3.2	3.6	6.8	1.8	1.8	3.6	1	0.4	1.4
SE ±		0.97	1.364	1.463	0.49	0.49	1.66	0.316	0.245	0.4

Table 7. Transferrable and non-transferrable embryos and unfertilised ova collected

Trial groups	Number of cows	Total embryos collected		Transferable embryo		Non transferable embryos			
						Morphologically abnormal		Unfertilised	
		No.	%	No.	%	No.	%	No	%
1	12	56	65.88	52	61.17	2	2.35	2	2.35
2	6	22	25.88	19	22.35	2	2.35	1	1.18
3	4	7	8.23	5	5.88	1	1.18	1	1.18
	22	85		76	89.41	5	5.88	4	4.70

Table 8. Percentage of transferable morulae and blastocysts

Trial groups	No. of cows	Total transferrable embryos		Transferable			
				Morulae		Blastocysts	
		No.	%	No.	%	No.	%
1	12	52	73.7	44	84.60	8	15.39
2	6	19	25.0	15	78.95	4	21.05
3	4	5	6.58	3	60.00	2	40.00
Total	22	76		62	81.58	14	18.42

Table 9. Distribution of embryos for freezing and thawing

Medium	Number of embryos	Freezing protocol	Number of Embryos	Dilution protocol	Number of embryos
10 % glycerol in PBS	24	a	12	1	6
				2	6
		b	12	1	6
				2	6
10 % glycerol + 1% BSA in PBS	24	a	12	1	6
				2	6
		b	12	1	6
				2	6
10 % glycerol + 20 % 1, 2 propanediol in PBS	24	a	12	1	6
				2	6
		b	12	1	6
				2	6

(a) Initial cooling at 1°C/min to –6°C, then at 0.3°C/min to –35°C.

(b) Initial cooling at 5°C/min to –7°C, then at 0.3°C/min to –30°C.

(1) 1 step – using 1 M sucrose phosphate buffered saline
(2) 4 steps – using decreasing concentration of cryoprotectant in PBS (7.5, 5.0, 2.5 & 0 percent)

Table 10. Number of embryos found to have normal morphology after freezing, thawing and cryoprotectant removal

Medium	Number of		Freezing protocol	Number of embryos		Cryo. removal protocol	Number of embryos		Normal embryos	
	M	B		M	B		M	B	M	B
10 % glycerol in PBS	20	4	a	10	2	1	5	1	2	1
						2	5	1	2	0
			b	10	2	1	5	1	1	1
						2	5	1	1	1
10 % glycerol + 1% BSA in PBS	20	4	a	10	2	1	5	1	4	1
						2	5	1	4	1
			b	10	2	1	5	1	3	0
						2	5	1	4	1
10 % glycerol + 20 % 1, 2 propane diol in PBS	20	4	a	10	2	1	5	1	1	1
						2	5	1	1	0
			b	10	2	1	5	1	1	1
						2	5	1	2	0
	60	12		60	12		60	12	26	8

M – Morula
B - Blastocyst
(a) Initial cooling at 1°C/min to –6°C, then at 0.3°C/min to –35°C.

(b) Initial cooling at 5° C/min to –7°C, then at 0.3°C/min to –30°C.

(1) 1 step – using 1 M sucrose phosphate buffered saline
(2) 4 step – using decreasing concentration of cryoprotectant in PBS (7.5, 5.0, 2.5 & 0 percent)

Table 11. Effect of media on the morphology after freezing

Medium	Embryos frozen	Found normal after freezing	Percentage
1	24	9	37.5
2	24	18	75.0*
3	24	7	29.17

* Significant at 5 per cent level (P<0.05)

Table 12. Effect of freezing protocols on the morphology

Freezing protocol	Number of embryos	No. of embryos found normal	%
1	36	18	50.0
2	36	16	44.4

Table 13. Effect of cryoprotectant removal on morphology

Protocol	Number of frozen	Embryos found normal	%
1	36	17	47.2
2	36	17	47.2

Table 14. Embryos found to have normal morphology after the culture

Medium	Number of cultured		Found normal	
	Morulae	Blastocyst	Morulae	Blastocysts
1	2	1	1	0
	2	0	1	0
	1	1	1	0
	1	1	1	0
2	4	1	2	1
	4	1	1	1
	3	0	2	0
	4	1	2	0
3	1	1	1	0
	1	0	0	0
	1	1	1	0
	2	0	0	1
	26	8	13	3

Table 15. Effect of media on the morphology after freezing and culturing

Medium	No. of embryos for culture	No: found normal	%
1	9	4	44.4
2	18	9	50.0
3	7	3	42.9

Table 16. Effect of freezing protocols on morphology after culture

Freezing protocols	No. of embryos for culture	No: found normal	%
a	18	8	44.4
b	16	8	50.0

Table 17. Effect of cryoprotectant removal on morphology after culture

Protocol	No. of embryos for culture	No: found normal	%
i	17	8	47.1
ii	17	8	47.1

Table 18. Values of glucose, AKP and ACP in the luminal fluid of the recipient cows

Sl. No.	Gl mg/100 ml	AKP KA units	ACP KA units
1	90.3	126.01	14.17
2	86.20	126.26	18.02
3	120.0	127.08	16.01
4	181.0	129.01	14.32
5	190.6	123.31	18.51
6	195.2	120.87	22.73
7	116.9	121.01	20.01
8	120.6	121.06	19.51
9	98.7	123.12	18.26
10	99.76	119.02	22.01
11	130.6	121.43	20.08
12	108.7	119.67	19.93
13	129.65	120.16	18.52
14	112.6	122.03	16.52
15	140.31	120.05	21.52
Mean & SE	128.0.75 ± 9.019	122.673 ± 0.788	18.675 ± 0.667

Table 19. Serum-progesterone levels in the recipients on day "O", 14 and 28 days after heat (ng/ml).

Sl. No. of animal	Progesterone		
	0 day	14th day	28th day
1	0.09	3.57	3.20
2	0.12	4.12	4.19
3	0.41	3.24	3.01
4	0.16	4.01	3.86
5	0.25	3.93	3.95
6	0.32	2.88	2.81
7	0.01	3.92	4.02
8	0.65	2.62	2.01
9	0.58	2.57	1.98
10	0.15	3.02	2.65
11	0.32	2.83	2.10
12	0.73	2.91	1.93
13	0.25	3.20	3.34
14	0.21	3.04	2.72
15	0.31	2.89	2.02
Mean	0.304	3.25	2.917
SE ±	0.054	0.136	0.21

Table 20. Serum – progesterone level of the recipient cows which showed subsequent first oestrum (ng/ml)

Sl. No. of animal	Progesterone		
	0 day	14th day	28th day
1	0.09	3.57	3.20
2	0.41	3.24	3.01
3	0.16	4.01	3.86
4	0.32	2.88	2.81
5	0.65	2.62	2.01
6	0.58	2.57	1.98
7	0.15	3.02	2.65
8	0.32	2.83	2.10
9	0.73	2.91	1.93
10	0.21	3.04	2.72
11	0.31	2.89	2.02
Mean	0.357	3.053	2.572
SE ±	2.14	0.42	0.627

Table 21. Serum-progesterone level of recipient animals which had not shown subsequent first heat (ng/ml)

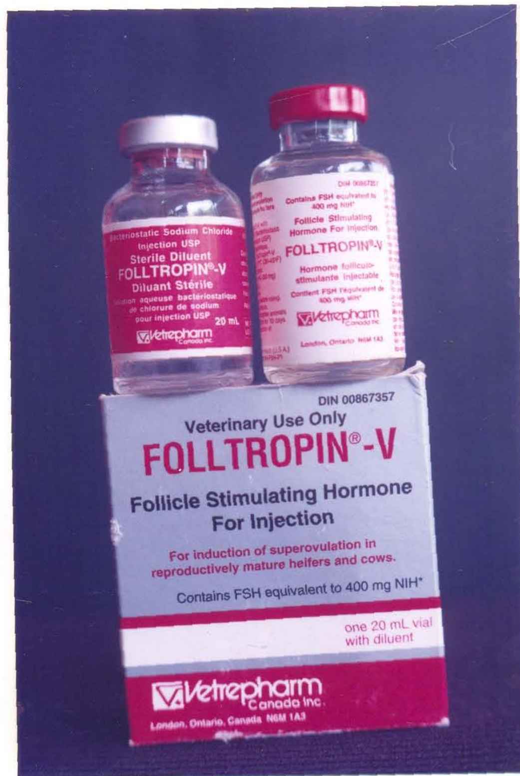
Sl. No of animal	Progesterone		
	0 day	14th day	28th day
1	0.12	4.12	4.19
2	0.25	3.93	3.95
3	0.01	3.92	4.02
4	0.25	3.20	3.34
Mean	0.157	3.793	3.867
SE ±	0.166	0.406	0.362

Table 22. Transfer of embryos after culture into the recipient animals

Sl. No. of animal	Transfer	Result
1	Morula	Negative
2	Morula	Pregnant
3	Morula	Negative
4	Morula	Negative
5	Blastocyst	EM *
6	Morula	Negative
7	Morula	EM*
8	Morula	Negative
9	Blastocyst	Negative
10	2 Morulae	Negative
11	Morula	Negative
12	Morula	Negative
13	Morula	EM *
14	Morula	Negative
15	Blastocyst	Negative

* Embryonic mortality suspected

Fig.1 FSH for superovulation



Bacteriostatic Sodium Chloride Injection USP
Sterile Diluent
FOLLTROPIN®-V
Diluant Stérile
20 mL
Vetrepharm
Follicle Stimulating Hormone For Injection
FOLLTROPIN®-V
Vetrepharm
DIN 00867357
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FOLLTROPIN®-V
Follicle Stimulating Hormone
For Injection
For induction of superovulation in reproductively mature heifers and cows.
Contains FSH equivalent to 400 mg NIH*
one 20 mL vial with diluent
Vetrepharm Canada Inc.
London, Ontario, Canada N6M 1A3

Fig.2 Prosolvin – $PGF_2\alpha$

Intervet

Fig.3 Good quality morula

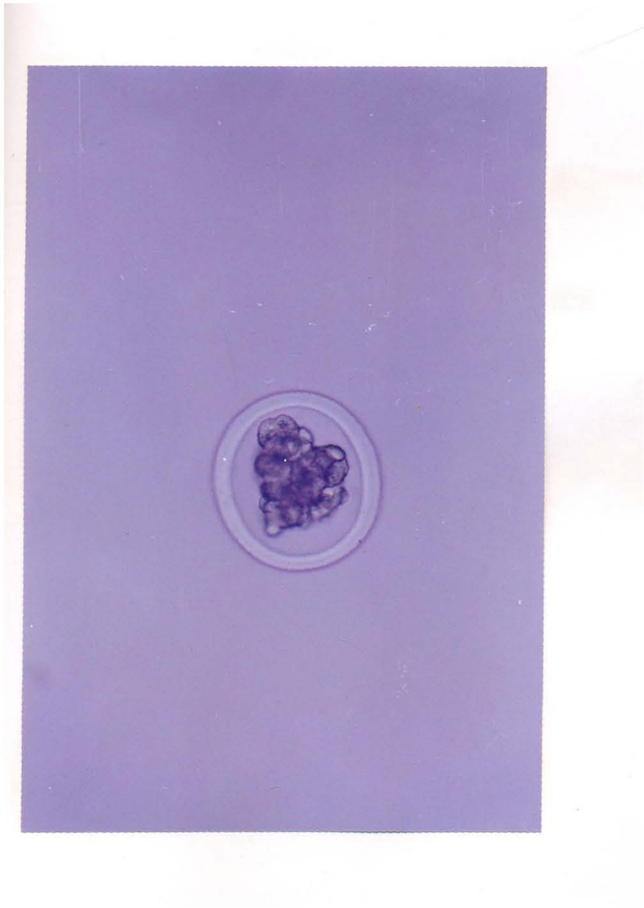


Fig.4 Good quality blastocyst

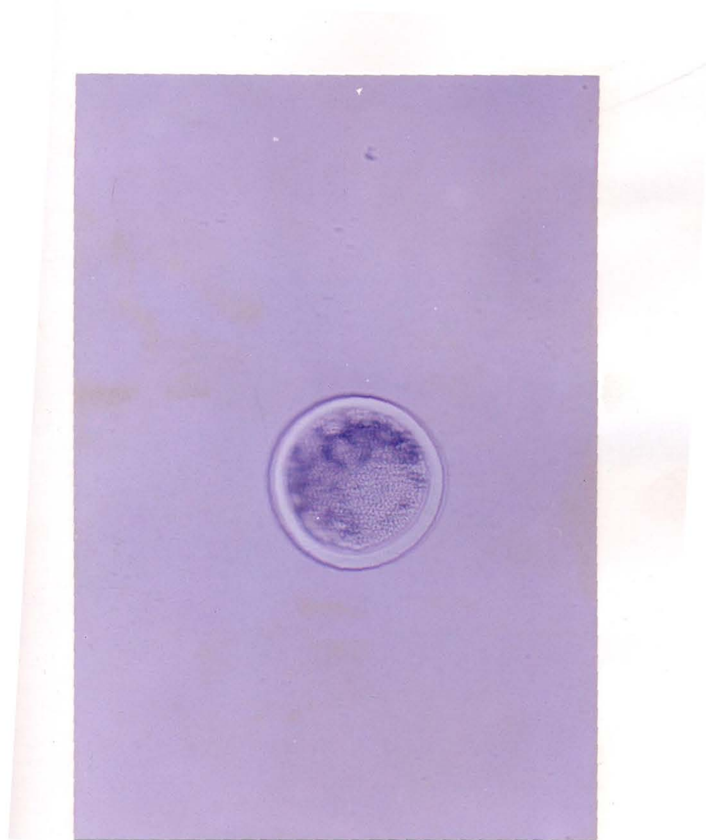


Fig.5 PERCENTAGE OF EMBRYOS RECOVERED

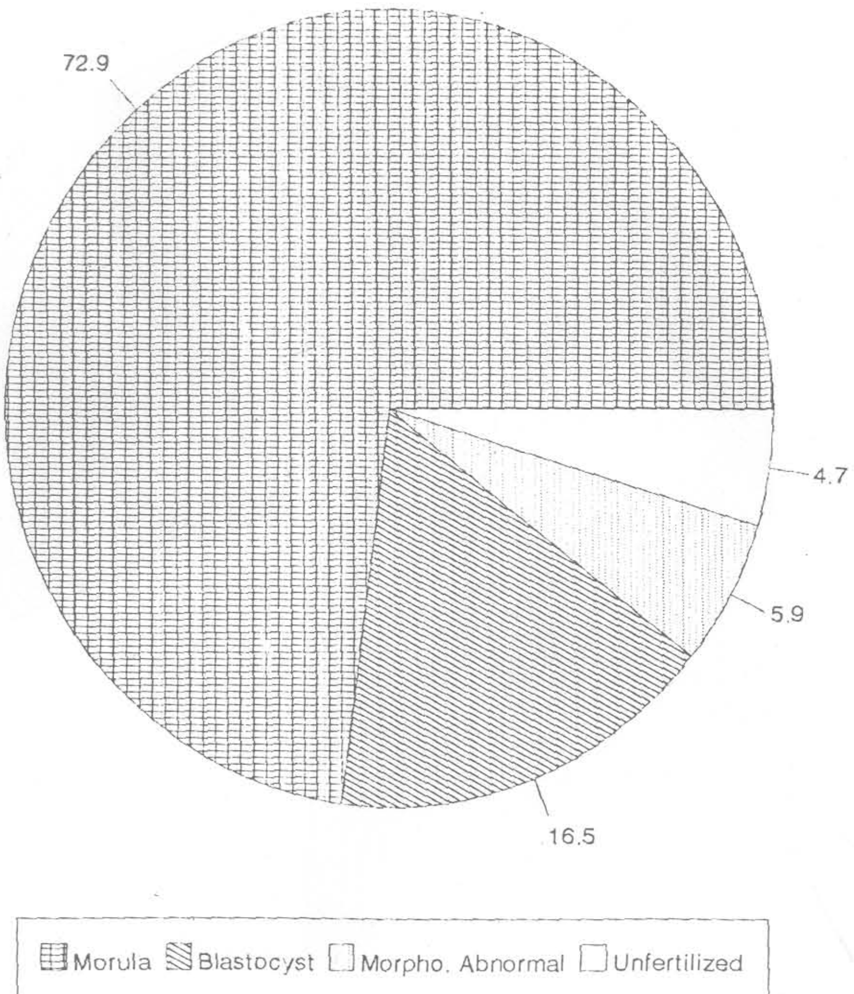
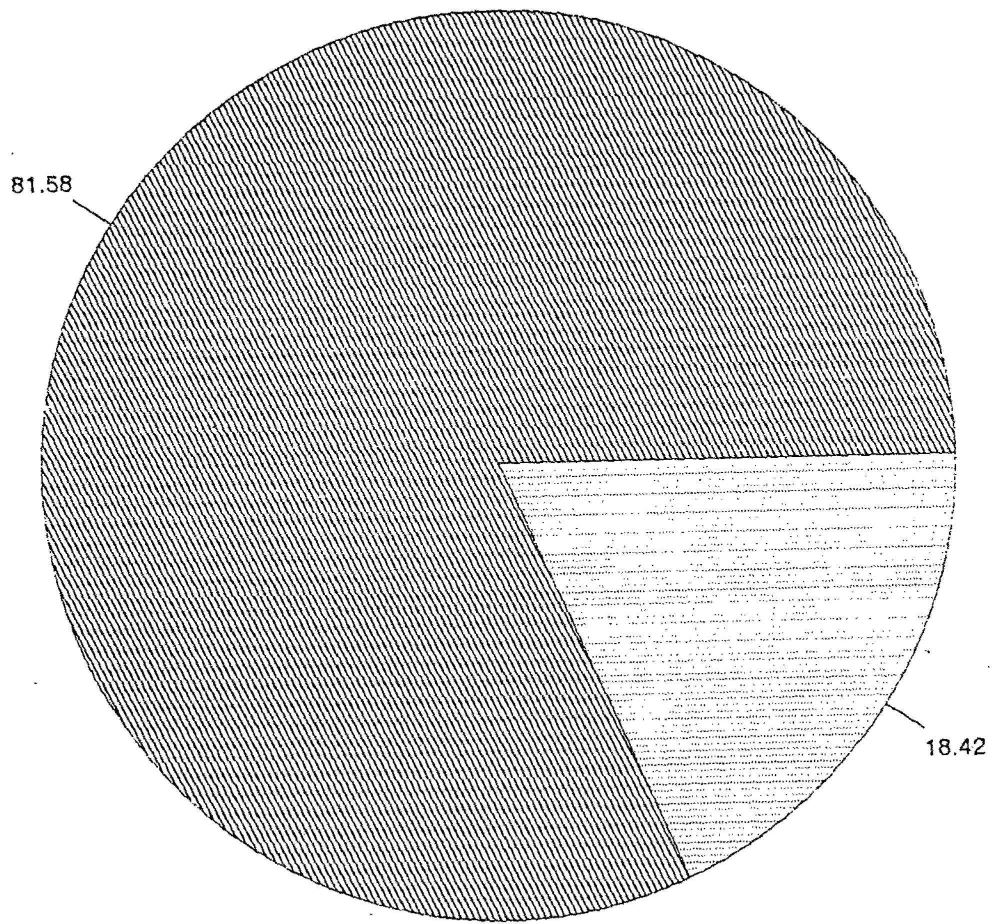


Fig.6 PERCENTAGE OF TRANSFERRABLE EMBRYOS RECOVERED



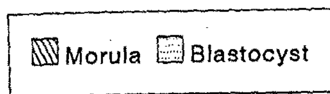


Fig.7 NUMBER OF TRANSFERRABLE EMBRYOS RECOVERED

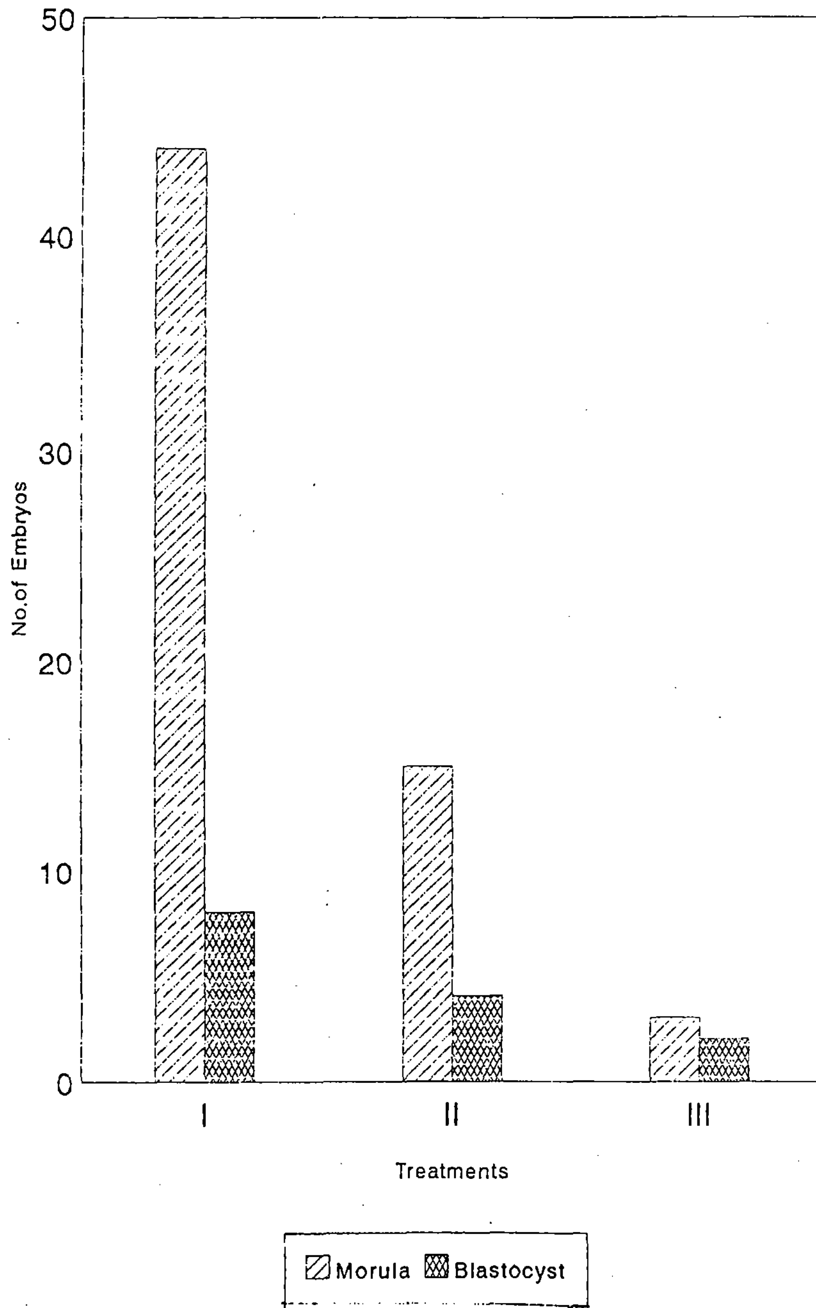


Fig.8 SUPEROVULATORY RESPONSE IN REPEAT TREATMENTS

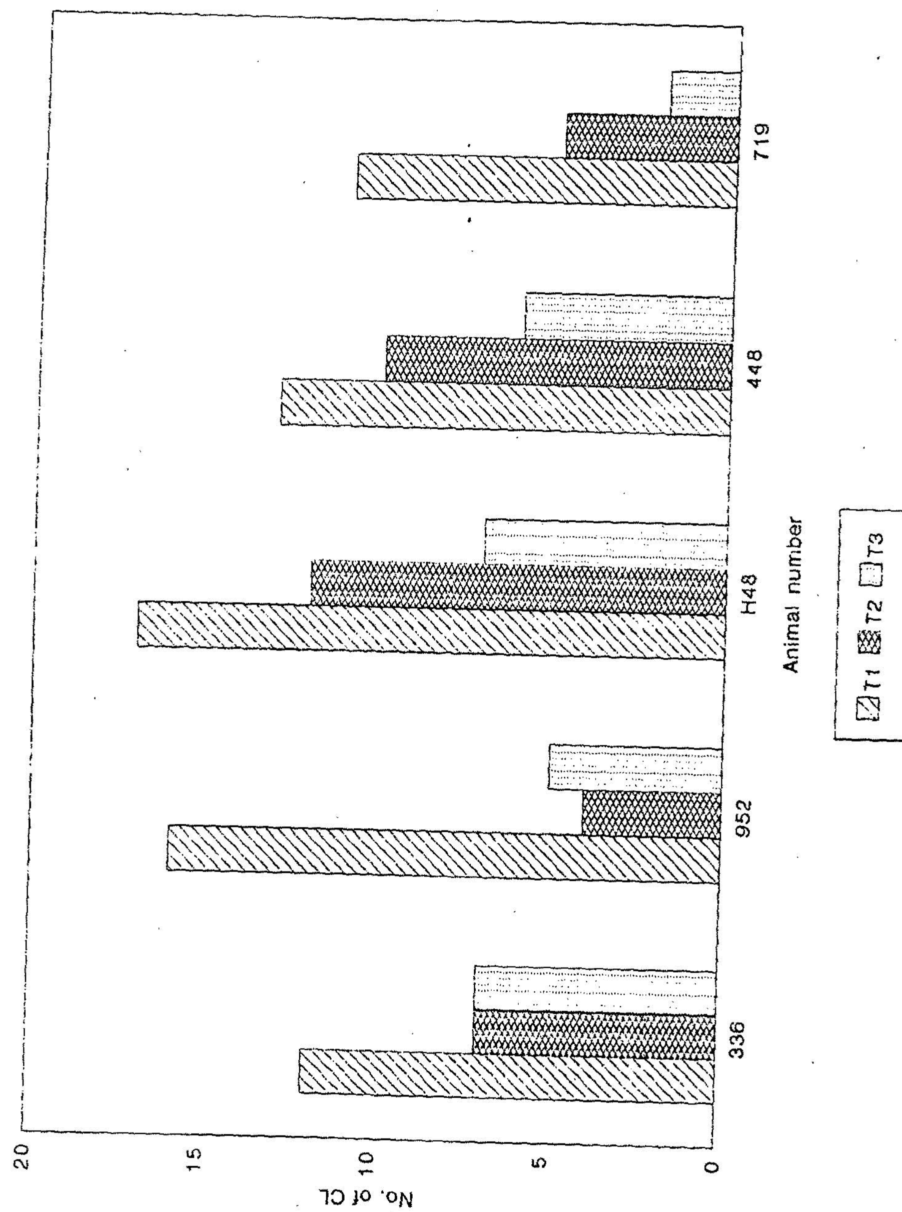
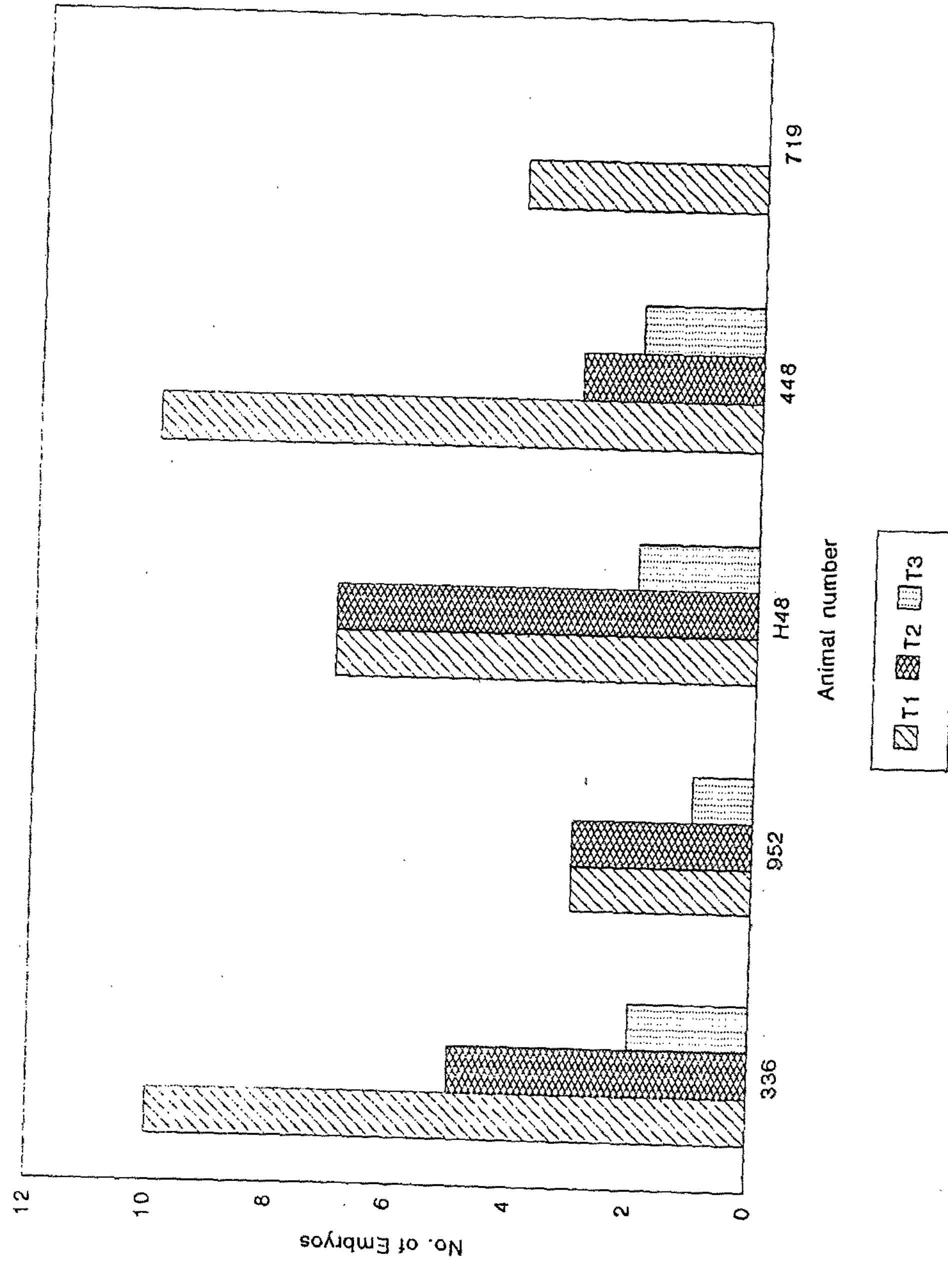


Fig.9 EMBRYO RECOVERY IN REPEAT TREATMENTS



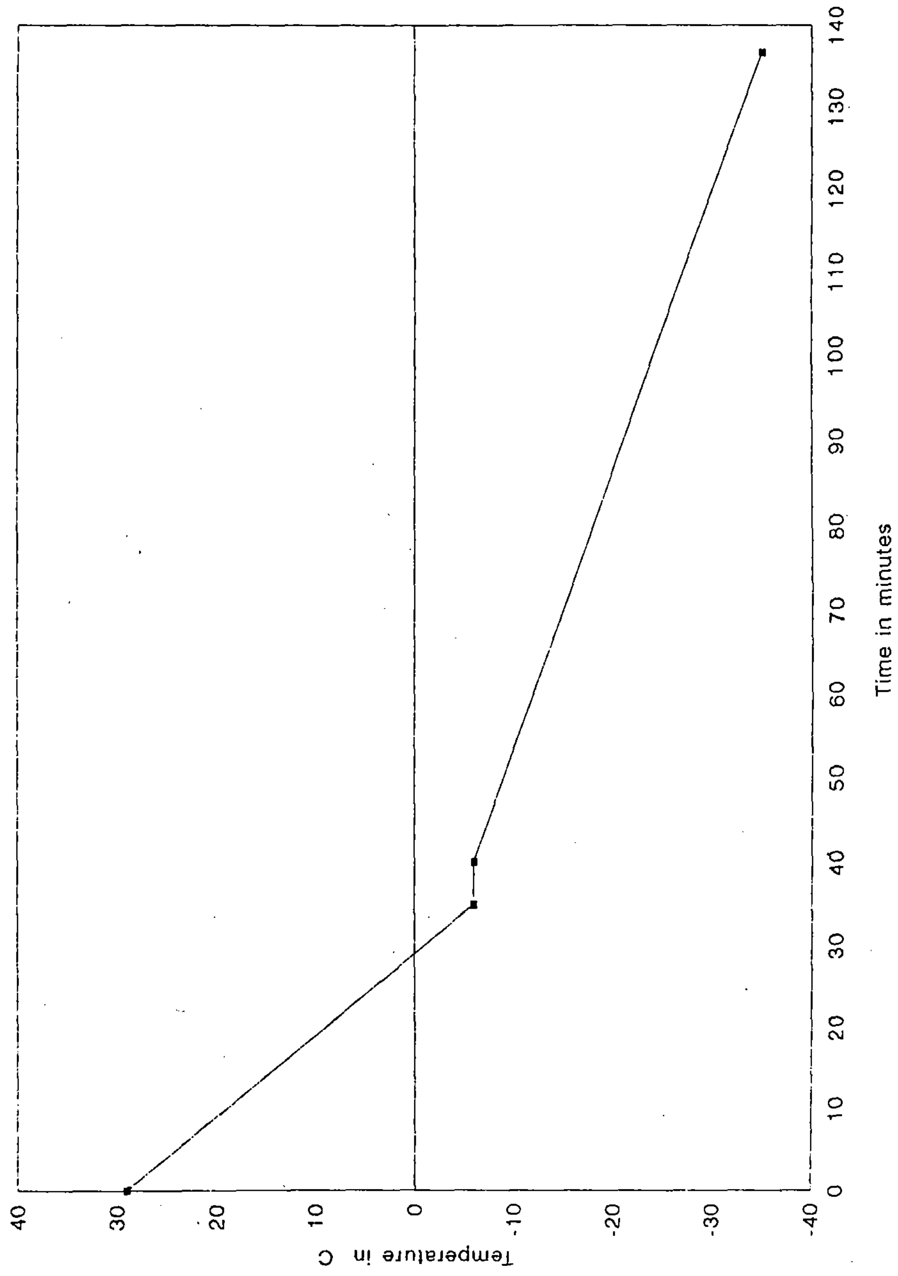


Fig.10 FREEZING PROTOCOL - 1

Fig.11 FREEZING PROTOCOL - 2

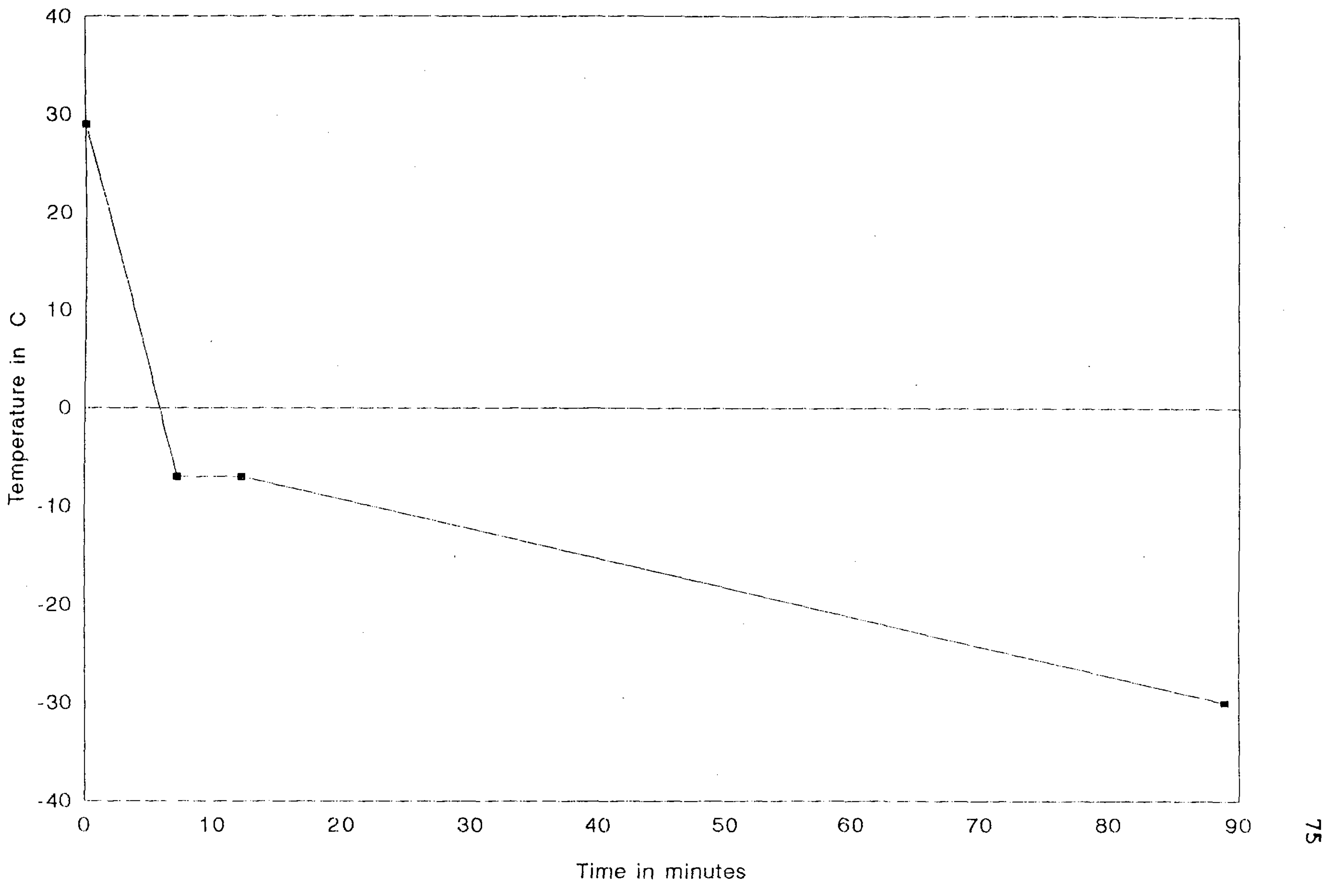


Fig.12 AVERAGE SERUM PROGESTERONE LEVEL IN THE RECIPIENTS

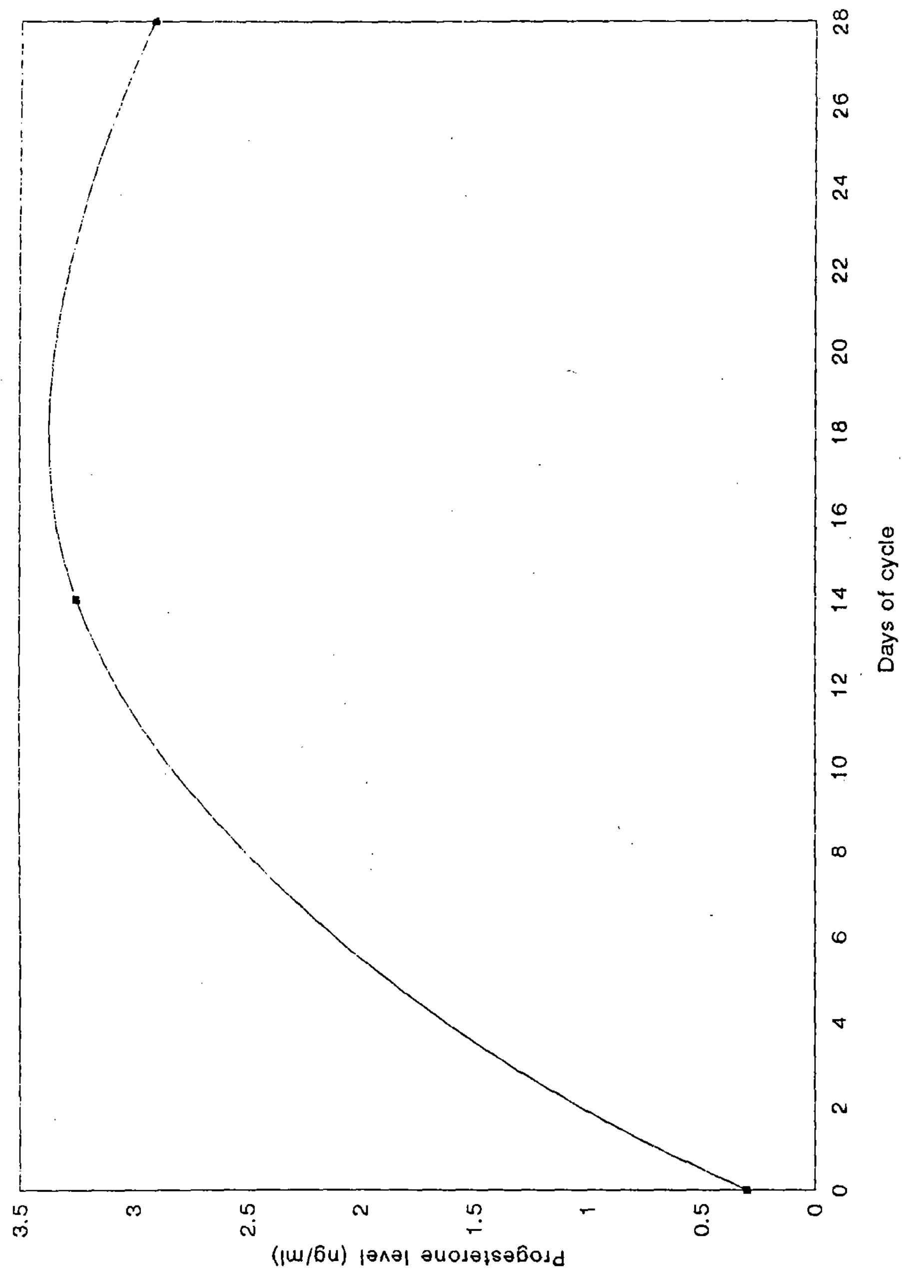


Fig.13 AVERAGE SERUM PROGESTERONE LEVEL IN THE RECIPIENTS
(11 ANIMALS)

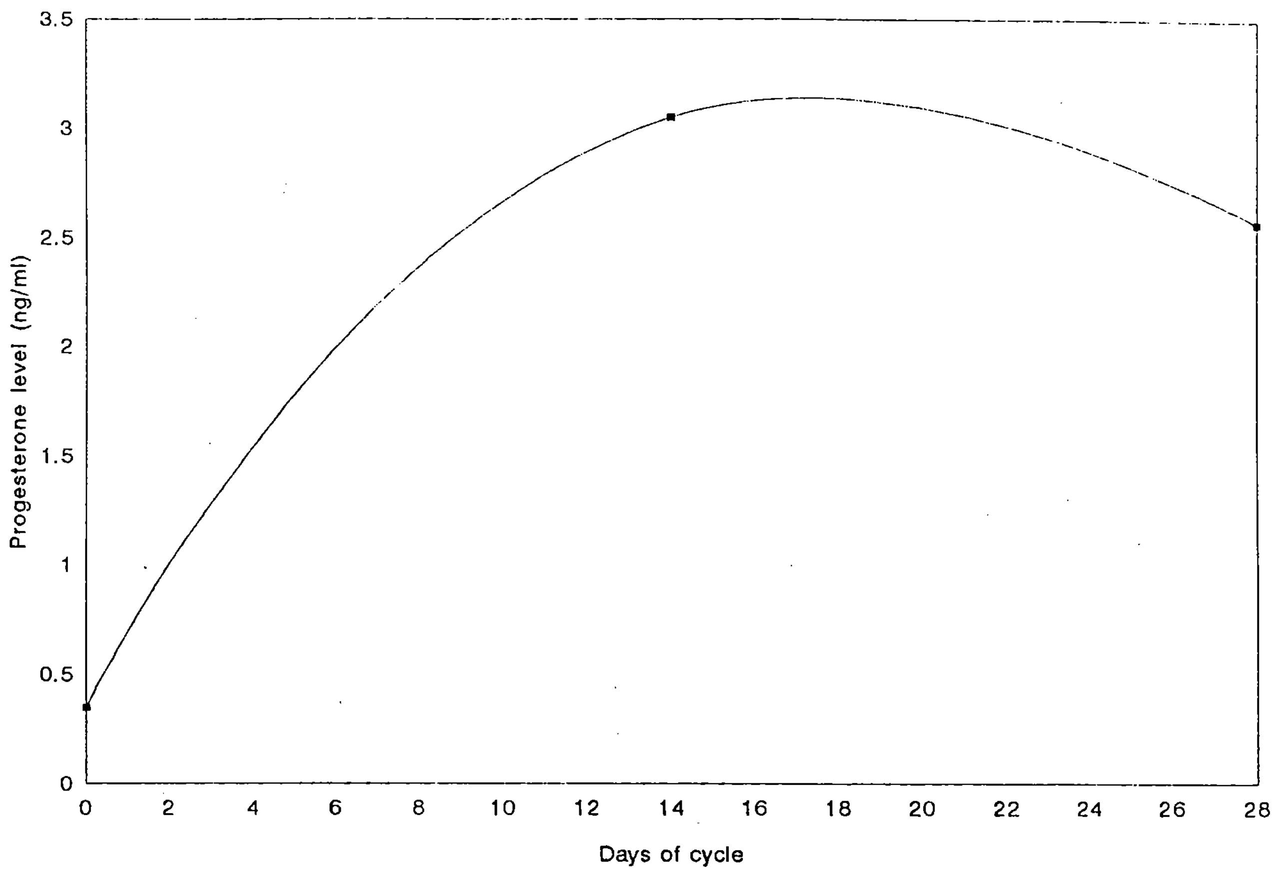
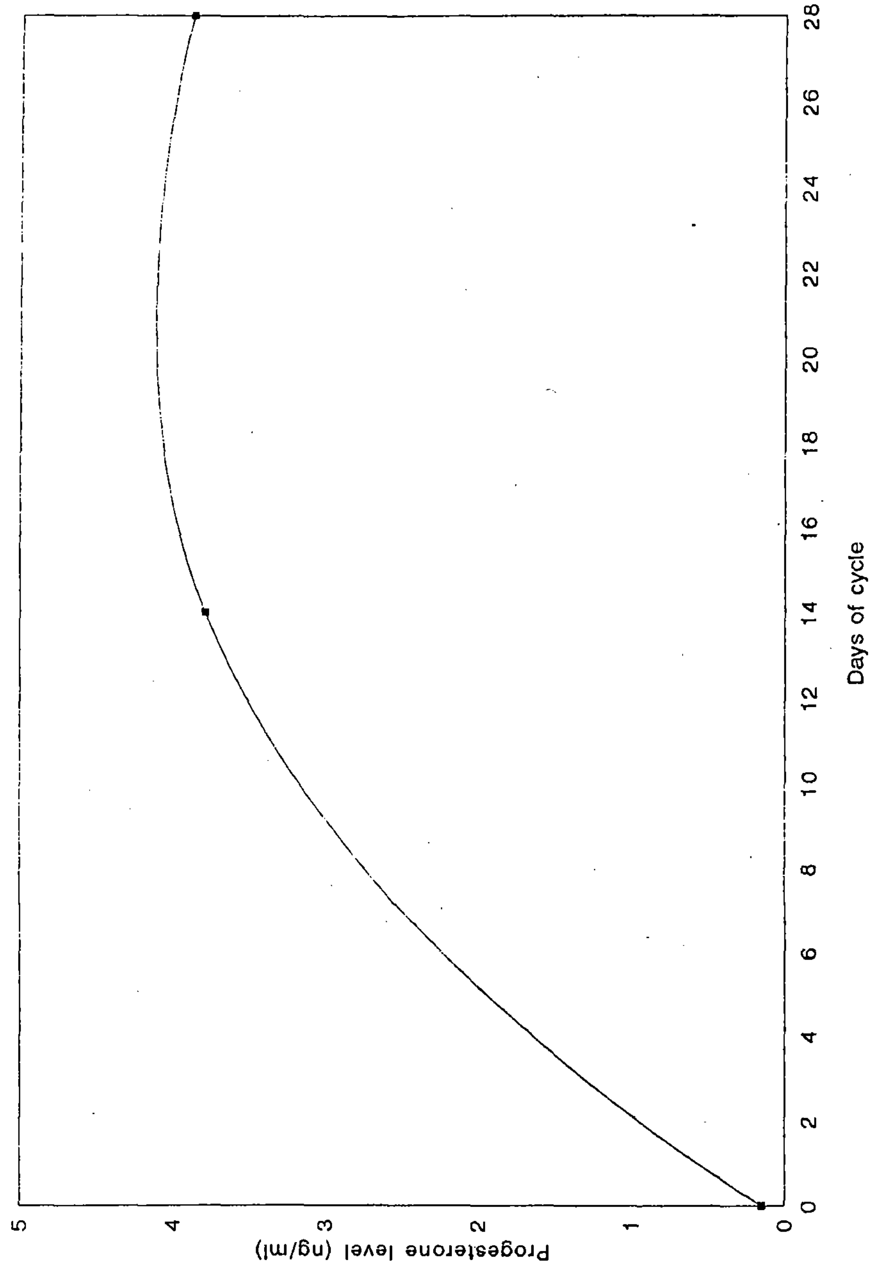


Fig.14 AVERAGE SERUM PROGESTERONE LEVEL IN THE RECIPIENTS (4 ANIMALS)



Discussion

DISCUSSION

The study was designed and conducted with the objective of evaluating the effect of Bovine Serum Albumin (BSA) and 1, 2 Propanediol on the morphology and viability of bovine embryos frozen under two protocols. Sixteen crossbred cows maintained at the Network Project on Embryo Transfer attached to the Department of Animal Reproduction, College of Veterinary and Animal Sciences, Mannuthy, were used for the study. All these animals were superovulated using FSH and prostaglandin $F_2 \alpha$ and embryos were recovered non surgically on day 7. Freezing was carried out under the conventional method as suggested by Massip *et al.* (1987) using two protocols. The embryos found normal after freezing and culture were transferred to synchronized recipient.

5.1 Selection of donors

In the present study sixteen crossbred cows of parity within the range of one to four were selected at random. These animals were apparently healthy and had normal reproductive status and were maintained under identical farm conditioins.

Hill *et al.* (1970) suggested that poor plane of nutrition affected the developments of follicles in mid luteal period. Drost (1986) opined that the animals selected for embryo transfer should have good reproductive status. Madan *et al.* (1993)

also were of the opinion that animals with history of high fertility make successful donors.

5.2 Superovulation

In the present study of induction of superovulation using FSH and PGF_2 α regime, out of sixteen animals, thirteen showed good response as evidenced from the development of number of corpora lutea. Two failed to show any response, while in one animal multiple follicles were found on both the ovaries with no sign of ovulation. Similar variability in ovarian response was reported and attributed to various factors such as environmental variations (Boland *et al.*, 1988; Xu *et al.*, 1988); animal to animal variations (Donaldson, 1986; Agarwal *et al.*, 1992; Hasler, 1992; Chauhan *et al.*, 1994) and the ovarian status of the donor at the time of hormone treatment (Monnieaux *et al.*, 1983; Armstrong, 1993).

FSH was given in divided and tapering dose regime for four days (Lindsell *et al.*, 1985; Saumande and Chupin, 1986; Xu *et al*, 1988; Pawshe *et al*, 1992; Sarma *et al.*, 1998) or in equal divided doses (Schiewe *et al.*, 1985; Thayer *et al.*, 1988) for four days. Single injection of FSH has also been reported by many workers (Bo *et al.*, 1994; Siddiqui *et al.*, 1996). In the present study FSH was given in equal divided dose regime for four days and had given satisfactory result, as 13 animals out of 16 had responded by superovulation.

Repeated superovulatory treatments in the present study showed a decreased response and embryo recovery rate. Repeated superovulations were reported to cause decreased response and embryo recovery rate (Donaldson and Perry, 1983; Bastidas and Randel, 1987; Totey *et al.*, 1992). The finding in the study was in accordance with the above workers. But Fielden and Haymann (1982) and Chauhan *et al.* (1994) reported no effect or inconsistent relationship between the number of superovulation trials and embryo recovery. Decreased superovulatory response with repeated treatments can be attributed to exogenous gonadotropins as reported by Jainudeen *et al.* (1966) or due to short recovery time after the previous superovulatory treatments (Lubbadeh *et al.*, 1980). Desaulniers et al. (1995) opined that attempts to improve superovulatory response were hampered by a number of reproductive disorders that were not evident in a study of unstimulated cycle.

5.3 Flushing efficiency and fluid recovery

In the present study flushing was successful in thirteen out of 16 animals and overall fluid recovery was 60 to 95 per cent. The difficulty in the fluid recovery due to cervical obstruction in the initial stage was not experienced in subsequent trails. The success of a flush is directly related to the success of fluid recovery (Gordon, 1996).

Brand *et al.* (1977) obtained 96 per cent fluid recovery, though there was difficulty in passing catheter, Sreenan, (1983) reported poor efficiency in flushing and

fluid recovery. Bleeding was also reported by Greve *et al.* (1977); Newcomb *et al.* (1978) and Manicham *et al.* (1990) while flushing.

5.4 Embryo recovery

Fifty six embryos were recovered from the initial flushings of 12 cows (mean 4.308 ± 0.865). Twenty two (mean 2.750 ± 0.861) and seven (mean 1.4 ± 0.40) embryos were obtained from the second and third subsequent repeat superovulatory trials, from seven and four flushings respectively. In the initial flushings of thirteen cows, though there was good fluid recovery of 80 per cent in one cow, no embryos could be recovered and the flushed fluid had only mucus and debris. In the second treatment, of the seven cows flushed, embryos were recovered only from six cows.

Rodrigues and Gregory (1986) recovered 64 per cent (139/215) transferable embryos from 31 donors and the average embryo per donor was 4.5. Siddiqui *et al.* (1996) reported that when the cows were superovulated second time the total as well as viable embryo recovery was reduced from 6.5 ± 1.65 and 4.66 ± 1.50 to 4.66 ± 1.75 and 2.23 ± 0.95 respectively. The mean number of embryos recovered was found to be 3.00 ± 0.29 (Sarma *et al.*, 1998) and they reported wide variations which was attributed to factors like technique of flushing and embryo searching.

5.5 Embryo Grading

In this study made, embryos were graded as transferable (89.41 per cent) and non transferable (10.5 per cent) depending on the morphological features. The embryos which were symmetrical with uniform size and colour with compact cells and intact zona pellucida were graded transferable. Embryos with loose blastomeres, debris and cracked zona pellucida were considered non transferable. Individual blastomeres coalesced forming a compact mass, occupying sixty to seventy per cent of the perivitelline space within the intact zona pellucida were graded as morula and those which revealed a fluid filled cavity or blastocoele were regarded blastocysts (81.58 per cent morula and 18.42 per cent blastocysts).

Morphological evaluation was widely used to determine embryo quality (Shea *et al.*, 1976; Elsden *et al.*, 1978; ; Shea, 1981; Wright, 1981; Schiewe *et al.*, 1985). System of classifying embryos into excellent, good, fair and poor categories appeared to be most reliable (Lindner and Wright, 1983). The parameters commonly used to evaluate embryo quality included shape, colour, number and compactness of cells, size and number of extruded or degenerated cells. Shea (1981) reported that better appearing embryos had higher survival rate, and the embryos which looked above average in appearance and rated as 4 had 71 per cent pregnancy when transferred fresh. Lindner and Wright (1983) were of the opinion that embryo quality was to be a more accurate predictor of success and the stage of embryo development appeared to have little effect on pregnancy rate. Excellent quality embryos when transferred fresh

had produced 45 per cent pregnancy while fair quality produced only 27 per cent. However the reproductive status, effect of sinchronisation, technique of embryo deposition, tolerance of the recipients also contribute to higher pregnancy rate.

5.6 Freezing

The aim of embryo storage in vitro is to preserve the embryo in a viable condition from which it may be revived after a short or long period to continue its normal development within the cow.

For the long term storage, embryos are frozen to −196°C in liquid nitrogen. This enables animal breeding centres to carry out a wider range of stocks and to store stock not in immediate use, thereby saving space and money. Inbred strains, mutation and special genetic combinations can be preserved. Genetic pedigree standards can be established and checked for genetic drift in subsequent generations (Hafez, 1993).

The biophysical principles that apply to cryopreservation of living cells and tissues also apply to cryopreservation of embryos. The preservation of viability after cooling is an essential pre-requisite for successful storage at low temperature. There are two approaches to the cryopreservation of embryos. The first is slow freezing (Whittingham *et al.*, 1972) and the second is vitrification as suggested by Luyet (1937). The factors primarily responsible for freeze injury to cells are ice formation within the cells and solution effects. The cell surface is found to be the main site of freeze injury (Friedler *et al.*, 1988). Permeating as well as non permeating additives have been

used successfully in the cryopreservation of embryos and ova. Permeating agents, in addition to protection from solution effects by their colligatie properties, also protect against injury from ice crystals. Addition of permeating agents to the medium decreases the temperature at which ice nucleation occurs within the cells.

The cryoprotectie effect of glycerol was first described by Polge et al. (1949). They had also reported the protective effect of propylene glycol (1,2 propanediol). Propylene glycol has great stability at sub zero temperature. It has been used in combinations with other agents in an attempt to minimise osmotic injury and specific toxicities. Successful cryopreservation usually requires optimization of each step of the procedure to account for the size, permeability and physiological characteristics of the constituent cells.

The freezing medium should contain either bovine serum albumin (BSA) or blood serum (often from fetal calves) that has been inactivated by treatment at 56°C for 30 min to remove thermolabile, an embryo toxic factor. BSA is added usually at 0.3 to one per cent (Hafez, 1993). It also act as a source of protein for embryo growth and membrane stabilization (Drost, 1986). Bovine serum albumin (BSA) was substituted for Newborn calf serum in 1.5 M glycerol solution used for freezing bovine embryos (Leibo, 1985). Pettit (1985) suggested that DPBS containing 0.4 per cent BSA and 10 per cent glycerol gave better results. Similar observations were also reported by Bondioli *et al.* (1984) and Takeda (1985).

Halasz and Collins (1984) have shown that the cryoprotectant induced injury could significantly be reduced by combining two agents of low toxicity. Massip *et al.* (1987) also reported better results when combination of glycerol and propanediol were used.

In the present study the effect of both BSA and propanediol was tried in freezing. Ten per cent glycerol in phosphate buffered saline was used as the primary freezing medium (medium 1). The second medium was prepared by the addition of one per cent BSA and the third medium by adding 20 per cent 1,2 propanediol. The study revealed that the medium containing 1 per cent BSA and 10 per cent glycerol in phosphate buffered saline had given better result. These observations are in agreement with the findings of Leibo (1985), Pettit (1985) and Drost (1986). However, the combination of glycerol and propanediol did not show any significant difference.

Most of the morula stage embryos are reported to have been killed while cooling rapidly to 0°C (Wilmut *et al.*, 1975). Vast majority of the bovine embryos have been cryopreserved using controlled freezing technique. Mazur (1977b) stated that optimal freezing rate for a given tissue depends on its relative tolerance to damage from ice crystal formation and toxicity from solution effects. Slow freezing prevents large ice crystal formation but leads to increased solution effects, whereas fast freezing prevents solution effect but cause large ice crystal formation. Most acceptable freezing rates reported were 1°C/min or 2°C/min to –6°C or –7°C and then at 0.1°C or 0.3°C/min to plunging temperature (Bielanski *et al.*, 1984; Slade *et al.*, 1984; Fehilly *et al.*, 1985; Leibo, 1985;

Maria *et al.*, 1985; Megahad, 1995). Most methods for cryopreservation of embryos which employ a controlled cooling process use seeding of ice crystals at a temperature of about –6°C to avoid excessive super cooling of the embryos. Following the formation of ice crysals in the medium, there is an increase in temperature due to the release of latent heat of fusion. This temperature increase and the change in osmolarity that occur require a holding period after seeding usually about five min for the equilibration of temperature (Nieman, 1991).

Two freezing protocols were compared in the present study. In the first protocol, the initial rate of cooling was at the rate of 1°C/min from 29°C to –6°C. After seeding and holding for five minutes the rate was at 0.3°C/min to the plunging temperature. In the second protocol the initial rate was at 5°C/min to –7°C and then at 0.3°C/min to the plunging temperature. No significant difference was noticed between the two protocols on the morphology of embryos after freezing, except that the second protocols was less time consuming.

When slow rates of cooling were used, the rates of embryo survival was determined by the break point temperatures at which cooling was terminated prior to immersion in liquid nitrogen (Willadsen, 1977; Whittingham *et al.*, 1979 and Bielanski *et al.*, 1984). Schmidt *et al.* (1985) reported no variation in the survival rate of bovine embryos when the plunging temperatures were –30°C, -35°C or –80°C, whereas Massip *et al.* (1987) reported a significant effect of plunging temperature on *in vitro*

survival of cow embryos. In this study the temperatures prior to transfer into liquid nitrogen were –35°C and –30°C respectively, for the first and second protocols.

5.7 Thawing

Damaged zona pellucida were often observed in frozen-thawed bovine embryos after liquid nitrogen storage. When straws were thawed in air or exposed to air (5 sec) before thawing in 38°C water, damage was minimal. When thawing was accomplished entirely in water (especially at 60°C) the number of damaged zona pellucida increased (Takeda, 1985). In the present study thawing was carried out in a water bath at 37°C for 20 sec.

5.8 Cyoprotectant removal

Because of toxicity at warmer temperatures, cryoprotectants are to be extracted before thawed cells are grown in culture. The two methods commonly employed to remove the cryoprotectants from embryos are dilution in serial steps and the transfer to a solution of a non-permeating agent. Sucrose solution controls the degree of swelling during the cryoprotectant removal by counteracting the initially high intracellular osmolarity of the cryoprotectant permeated cells as suggested by Leibo (1985), Schneider and Mazur (1984). When embryos were frozen and placed directly in PBS after thawing blastomeres were found to swell and desentigrate (Willadsen et al., 1978).

Massip and Van Der Zwalmen (1984); Nieman (1985) and Van Waglendonk-Leeuw *et al.* (1994) reported no significant difference when one step dilution or dilution in serial steps were used for the cryoprotectant removal. On the other hand Agarwal and Polge (1989b) reported that embryos in serial dilution exhibited higher survival rate as compared to the one step dilution. As a measure to compare the observations, one step dilution and a four step serial dilutions were tried in the present study. No significant difference was observed between the two procedures in this study. The second method was comparatively less time consuming and the chance of loss of embryo during the process was low.

5.9 Culture

Trounson *et al.* (1976) reported that cow morulae were capable of normal development in PBS medium supplemented with serum. Smith *et al.* (1986) indicated that HAMS F-10 medium was found to be superior to PBS in respect of growth, survival and hatching of bovine embryos during 72 h culture at 37.5 °C. Bilton and Moore (1979) reported that a short period of culture (12-18 h) provided a rapid assessment of viability. Don Roger (1984) stated that it was highly desirable to predict the probability of survival of embryos prior to transfer and suggested assessment of metabolic activity only as an alternative to morphologic assessment. Although culturing of embryos in vitro after thawing helped to evaluate their quality by morphological appearance, it did not improve their viability (Maria, 1985). A short period of 24 h culture was tried in the present study using PBS enriched with 4 per

cent BSA as the culturing medium, at 37°C and 5 per cent CO_2 tension. Following their culture, only 16 of the 34 embryos cultured had normal morphology. The lower result observed could be attributed to variation in the technique in handling of embryos during the freezing, thawing and their *in vitro* culture.

5.10 Recipients

Recipient cows were selected from the University Livestock Farm and from among the animals brought to the AI centre. Madan *et al.* (1993) opined that consideration for selection of recipients was important. The animals should have sound reproductive tract with ovaries of normal size, shape and cycle length, proven fertility and free from disease which affect mothering ability. The cows which had normal oestrous cycle with normal breeding history and parity in the range of one to four were only selected as the recipients in this study.

5.10.1. Biochemical study

The examination of cervical mucus of cows in oestrus helps in diagnosis of the stage of heat and in differentiating normal and repeat breeding cows (Wani, 1976). The significance of various biochemical constituents of cervical and uterus fluids has been well recognized as their deficiencies or excess adversely affect the viability and fertilizing ability of sperms. The importance of uterine secretions as a medium for sperm capacitation, embryo implantation and as an embryotrophic to developing conceptus has lead to explore the biochemical constituents. Nutritive material primarily glycogen is gradually stored in the uterine epithelium and muscularis under

the influence of oestrogen (Old and Van Demark, 1957) to be metabolized and utilised by implanting blastocysts and to maintain dynamic status of uterus. Acid phosphatase level might be beneficial for hydrolysing phosphomono esters, and then provide energy in the form of phosphatase for normal reproductive functions. The decreasing level of alkaline phosphatase favour folliculogenesis and further enhance change of conceptus in fertile cows (King, 1971, Devaraj, 1983, Sinha et al., 1986). Mehta et al. (1989) reported increased activity of alkaline phosphatase from estrus to 1st trimester of pregnancy in normal cycling cattle. Cervical mucous of normally conceiving cows had non significantly higher inorganic phosphorus and glucose than the cows which failed to conceive (Sood et al., 2000). Higher ACP level on day of estrus was reported to be significantly higher in repeaters (Sharma and Tripathi, 1985; Shukla, 1989). Acid phosphatase level was useful index for fertility assessment with regard to the oestrogen level (Saphale et al., 1994). In the present study, the recipient cows were healthy with normal reproductive cycles and the values of glucose. ACP and AKP were within normal range. These values could not be correlated to the lower pregnancy rate obtained.

Agarwal et al. (1977) reported significant difference on day 18-21 for the overall progesterone level. The level was higher on day 28 for all the cows which conceived but the difference was not significant. Hasler et al. (1980) opined that the most suitable level for pregnancy diagnosis would vary probably because of variations in assays among the laboratories and type of management programme. The level of

serum progesterone was found to be between 0.01 and 4.19 ng/ml on day 0 to 28 respectively in the present study. Statistical analysis (t-test) between the four animals which did not show heat in the first cycle and the rest 11 cows which came in oestrum revealed significant difference between mean values for days 14 and 28 while it was not significant for day 0.

5.10.2 Viability study

Sixteen embryos found morphologically normal were transferred to 15 synchronised recipient cows. In one cow two embryos were transferred. Of these 15 recipients, one was confirmed pregnant on examination after 60 days of transfer. Three cows showed the presence of CL when examined 30 days after the transfer. Of these animals two evinced oestrus 34 and 35 days after the transfer while the third showed signs of oestrum, 45 days after transfer.

Yamashina(1989); Hahn (1991); Holey *et al.*, (1992) and Krugar et al. (1992) reported 60 to 83.3 per cent pregnancy rate for fresh embryo transfers. Takeda *et al.* (1985) obtained 54 per cent while Neimann *et al.* (1992) reported 62.5 per cent for fresh transfers. Jordt and Lorengini (1988) obtained only 20 per cent while a higher pregnancy rate of 30 per cent was reported by Subramanian *et al.* (1991); Subramanian and Devarajan (1991); Totey *et al.* (1991a); Nair (1992) and Lee *et al.* (1993). . Manicham *et al.* (1990) and Javed and Ullah (1995) reported zero per cent pregnancy rate for fresh transfers.

Pregnancy rate with deep frozen and thawed embryos were found to be lower (Schiewe *et al.*, 1985; Wurth *et al.*, 1994). Elsden *et al.* (1982) reported 74.3 per cent and 44.5 per cent pregnancy rate for fresh and frozen embryo transfers. Similarly, Gustafson *et al.* (1994) reported significantly lower pregnancy rate (8 per cent) for frozen thawed embryos while for fresh transfers 30 per cent was obtained. Sungur and Yurdayadin (1994) reported 31 per cent pregnancy (6/19) for frozen embryos transferred. Nigro and Burry (1995) had opined that the breed of the embryo had significant effect on pregnancy rate.

The low rate of pregnancy obtained in the present study could be attributed to the recipient tolerance or due to poor survival rate in reproductive tract by the embryos. Further study is to be carried out in larger number of animals.

Summary

SUMMARY

The objective of the present study was to compare the effect of bovine serum albumin and 1,2 propanediol on the morphology and viability of bovine embryos frozen under two different freezing and dilution procedures.

Sixteen healthy crossbred cows kept under the same conditions of feeding and management, maintained in the Network Project on Embryo Transfer attached to the Department of Animal Reproduction, College of Veterinary and Animal Sciences, were used for the study. These cows were superovulated using Folltropin-V given at 50 mg twice daily for four days commencing on the day 11 of the cycle. Prosolvin, 15 mg was administered after 48 h of FSH initiation. These animals were inseminated twice daily for two days with good quality chilled semen collected from the bulls maintained in the AI centre attached to the Department.

Out of the 16 cows superovulated, 13 showed good response. While in one cow, multiple follicles were seen on both the ovaries without any evidence of ovulation, two cows did not show any response. The right ovaries showed better response with a range of three to ten CL (Mean 5 ± 0.747, 3.375 ± 0.596 and 2.8 ± 0.496 for 1st, 2nd and 3rd treatment respectively). Embryos were recovered nonsurgically on the day 7, using Dulbeccos phosphate buffered saline medium.

A total of 56 embryos were recovered in the 1st treatment (Mean 3.5 ± 0.822) of 16 cows. In the second and third treatments total number of embryos recovered were

were 22 and 7 with a mean of 2.75 ± 0.861 and 1.4 ± 0.4 respectively. In one cow, no embryos could be recovered, though there was 80 per cent flushing recovery. The flushed medium contained only mucus and debris. The collected embryos were graded as transferable (76) and non transferable (9). While 62 embryos were morulae, 14 were of early blastocyst stage in the transferable group.

Among the non transferable, five were degenerated ones and four unfertilized. In the first superovulatory treatment of cows, 52 transferable (44 morulae and 8 blastocysts) embryos were recovered. Nineteen embryos of transferable grade (15 morulae and 4 blastocysts) were only recovered from the second trial, flushing six cows. In the third treatment, from five flushings, only five (3 morulae and 2 blastocysts) transferable embryos could be recovered. A decrease in the superovulatory response and embryo recovery was seen in repeat treatments and flushings. The fluid recovery ranged, on the whole, from 60 to 95 per cent.

Seventy two (60 morulae and 12 blastocysts) embryos were selected for the freezing trials, divided into three groups having 24 (20 morulae and 4 blastocysts) each and were allotted to three different media. The first freezing medium had a composition of 10 per cent glycerol in phosphate buffered saline, the second medium had 10 per cent glycerol and 1 per cent BSA in PBS, and the third contained 10 per cent glycerol and 20 per cent 1, 2 propanediol in PBS. Two freezing protocols were used for freezing embryos of each group, and each protocol had 12 embryos (10 morulae and 2 blastocysts). In the first protocol embryos were cooled from the room

temperature to –6°C at a rate of 1°C/min and after a pause of 5 min the rate was reduced to 0.3°C/min to –35°C and transferred immediately into liquid nitrogen. The initial rate of cooling was 5°C/min to –7°C in the second protocol and after a pause of 5 min the rate was 0.3°C/min to –30°C before plunging into liquid nitrogen. Thawing was done at 37°C in a water bath for 20 sec, after 30 days of preservation. The cryoprotectants were removed in two different methods. In the first method, a four step dilution with decreasing concentrations of cryoprotectants in PBS was used. 1 M sucrose phosphate buffered saline was used at room temperature for 20 min in the second method. Embryos after washing in PBS were examined for morphology. After freezing 72 embryos, only 34 (26 morulae and 8 blastocysts) embryos were seen to have normal morphology, and were subjected to 24 h culture at 37°C and 5 CO2 pressure using phosphate buffered saline enriched with 4 per cent BSA. Only 16 embryos (13 morulae and 3 blastocysts) revealed normal morphology after the culture.

A total of fifteen recipient animals were selected. These animals were watched for oestrum and the luminal fluid was collected aseptically. The glucose, acid phosphatase and alkaline phosphatase were estimated using O-Toludine, modified King's and Kind and Kings methods respectively. Serum was collected from the recipient cows on day of heat, 14 and 28 days after the heat and progesterone estimated by ELISA method.

Significant difference was seen between the freezing media 1 and 2 and media 2 and 3 on the morphology of embryos after freezing, thawing and the cryoprotectant

removal. No significant differences was seen between the freezing protocols and dilution methods on the morphology of embryos after freezing. After the culture, no significant differences were seen in the effects of media, freezing protocols and the dilution methods on the morphology of embryos.

The values of glucose, acid phosphatase and alkaline phosphatase ranged from 86.2 to 195.2 mg/100 ml (mean 128.075 ± 9.019); 14.17 to 22.01 KA (mean 18.675 ± 0.667) and 119.2 to 129.01 KA (mean 122.673 ± 0.788) respectively. The average serum progesterone values of 11 cows which showed subsequent first heat on days 0, 14 and 28 days after oestrum were 0.357 ± 2.14 , 3.053 ± 0.420 and 2.572 ± 0.627 ng/ml respectively. In four cows which did not show estrus the values were 0.157 ± 0.166, 3.793 ± 0.406 and 3.867 ± 0.362 ng/ml respectively.

Sixteen embryos were transferred non surgically to 15 recipient animals. Among these 11 evinced oestrum subsequently two cows were inostrum 34 and 35 days after transfer while third one showed oestrum 45 days after the transfer. One cow was confirmed pregnant 60 days after transfer.

Conclusion

From the present study made on the morphology and viability of embryos frozen in media containing BSA and propanediol, the following conclusions could be drawn.

1. Folltropin-V given at equal divided dose twice daily for four days had given a good superovulatory response.

2. Higher percentage of embryos retained normal morphology when freezing medium containing BSA was used.

3. Both freezing protocols were found have similar effect on the morphology and viability of embryos after freezing, since in both methods, the number of normal embryos recovered were almost equal.

4. Serial step wise and one step method of cryoprotectant removal did not affect the morphology of embryos, frozen in different protocols using different media.

5. Irrespective of the protocols of freezing and thawing, cryoprotectant media containing BSA will be best suited for embryo preservation.

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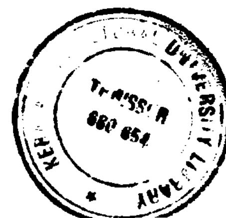

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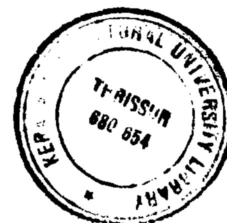

MORPHOLOGY AND VIABILITY OF BOVINE EMBRYOS FROZEN IN MEDIA CONTAINING BSA AND PROPANEDIOL

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ABSTRACT OF A THESIS

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ABSTRACT

The observation of the study was to compare the effect of Bovine Serum Albumin (BSA) and 1,2 Propanediol on the morphology and viability of bovine embryos frozen under two freezing and thawing protocols.

A total of sixteen crossbred cows, kept under identical conditions, maintained in the Network Project on Embryo Transfer attached to the Department of Animal Reproduction, College of Veterinary and Animal Sciences, Thrissur.

The animals were superovulated by Folltropin-V and Prosolvin, starting on the day 11 of the cycle. Of the 16 cows superovulated 13 showed good response. While two cows did not show any response, there were multiple follicles in both the ovaries without any evidence of ovulation in the third animal. A total of 85 (76 transferable and 9 non transferable) embryos were recovered from a total of 24 flushings from 13 cows, non-surgically on day 7. A total of 56 embryos (mean 3.50 ± 0.822) were recovered in the first treatment, from 13 flushings. In one cow, though, there was 80 per cent flushing, no embryos could be recovered. While 22 embryos (mean 2.75 ± 0.861) were recovered in the second treatment from 8 flushings, only 7 (mean 1.4 ± 0.4) embryos were recovered in the third treatment, from 5 flushings.

A total of 72 transferable (60 morulae and 12 blastocysts) were slected for the freezing trials. The embryos were divided into three groups with 24 (20 morulae and 4

blastocysts) embryos and assigned to three media. The first medium was FBS with 10 per cent glycerol, second PBS containing 10 per cent glycerol and 1 per cent BSA; the third medium was with a composition of 10% glycerol and 20% 1,2 propanediol in PBS. Two freezing protocols were used for freezing of the embryos. In the first protocol, with 12 embryos (10 morulae and 2 blastocysts), the initial cooling was at a rate of 1°C/min from room temperature to –°C and then at a rate of 0.3°C/min to –35°C, while in the second protocol the initial rate of cooling was at 5°C/min to –7°C and then at 0.3°C/min to –30°C before transferring to liquid nitrogen. Thawing was carried out at 37°C for 20 sec after 30 days of preservation. Cryoprotectants were removed by two methods, a four step-wise using decreasing concentrations of cryoprotectants in the first method and one step using 1M sucrose phosphate buffered saline in the second. Thirty four embryos (26 morulae and 8 blastocysts) found normal after freezing and thawing were subjected to culture for 24 h in PBS enriched with 4 per cent BSA at 37°C and 5 per cent CO_2 tension. Sixteen embryos (13 morulae and 3 blastocysts) were transferred to 15 recipient cows. While one cow was confirmed pregnant on examination 60 days after transfer, eleven cows returned to heat subsequently, two cows came to oestrus on days 34 and 35 respectively, after the transfer. The third showed oestrum on 45th day of transfer. The glucose, acid phosphatase and alkaline phosphatase values showed a normal range of 86.2 to 195.2 mg/100 ml; 14.17 KA to 22.3 KA and 119.02 to 129.00 KA (mean 128.075 ± 9.019, 18.675 ± 0.667 and 122.67 ± 0.788) respectively in the luminal fluid of the recipient animals. The average serum progesterone levels on day 0, 14 and 28 days after oestrus

in 11 cows which showed subsequent heat after the transfer were 0.357 ± 2.140, 3.053 ± 0.420 and 2.572 ± 0.627 ng/ml and that of the animals which failed to show oestrum were 0.157 ± 0.166, 3.793 ± 0.406 and 3.867 ± 0.362 ng/ml respectively.

While significant difference was seen between the freezing media I and II and II and III respectively on the morphology of embryo after the freezing and thawing, no significant differences were seen between the media I and III, between the freezing protocols and cryoprotectant removal procedures on the morphology of embryos frozen. No significant differences were noticed on the effect of the freezing media, freezing protocols and the cryoprotectant removal procedure after the culture on the morphology of the embryos.