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Superovulation and Embryo Recovery in the Guinea Pig

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Budapest
2013

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List of Acronyms

cDNA	complimentary deoxyribonucleic acids
CL	corpus luteum
eCG	equine chorionic gonadotropin
E.U.	European Union
˚C	degree Celcius
Cc	concentration
FSH	follicle stimulating hormone
FSH- R	follicle stimulating hormone receptor
g	gram
hCG	human chorionic gonadotropin
hMG	human menopausal gonadotropin
IU	international unit
IVF	*in vitro* fertilisation
LH	luteinising hormone
mg	mille gram
mL	mille litre
mm	mille meter
µl	micro meter
PMSG	pregnant mare serum gonadotropin
p.c.	post coitum
vs	versus
y	year

1. Introduction

Mice and rats are the most popular lab animals used in experiments across the world. They are also routinely used in studies about the reproductive system due to their large litter number, short gestation period and the ease of inducing ovulation. A large oocyte supply is also crucial for the preservation of genetic resources, conservation of endangered species, transgenesis, or cloning by nuclear transfer (Coello *et al.*). They are cheap and easy to maintain, while readily available in every country.

The guinea pig (*Cavia porcellus*) has been used as a laboratory animal since the 1700's (Terrill and Clemons, 1998) but has decreased in popularity in the lab throughout time. Despite being a rodent, the guinea pig is thought to be evolutionary different from mice and rats; there are many anatomical, reproductive and physiological differences which will be discussed later on.The reason why it has been used less frequently in the field of reproduction is the fact that they have a smaller litter number and a longer gestation period. It is also relatively difficult and time consuming to induce superovulation, especially since ovulation cannot be induced spontaneously as in mice and rats. Even so, there are many reasons why the guinea pig has been preferred for the study of human reproduction, all of which have motivated scientists across the world to work on improving methods of superovulation in order to collect larger amounts of oocytes or embryos for their studies.

Some of the reproductive similarities between humans and guinea pigs include a long gestation period, spontaneous ovulation, active corpora lutea, complete luteal and follicular phases, non-seasonal cyclic polyestrous, a fertile post-partum oestrous and a haemochorial placenta. While rats and mice will abort their young once the ovaries have been removed, the guinea pig will carry the young to term, making it a valuable model for the study of endocrine control during human pregnancy (Terrill and Clemons, 1998). These similarities support the use of the guinea pig as a more suitable model for the study of reproduction in humans, than mice and rats.

The aim of this study is to test out one of the methods proven to be successful in inducing superovulation and adjusting it to make it suitable for the use of common guinea pigs since

laboratoryspecific pathogen-free guinea pigs were unavailable. The protocol chosen belongs to Suzuki *et al.* (2000) which is the least expensive and least time-consuming when compared to Shi *et al.* (2000a,b)'s protocol using inhibin vaccine, and Kosaka and Takhashi (1989)'s protocol using pregnant mare serum gonadotropin (PMSG) after implantation of progesterone tubing.

The guinea pigs were given 5IU of human menopausal gonadotropin (hMG) on days 13, 14 and 15 post ovulation and the embryos were collected from the uterus on Day 4 post-coitum. An important difference between mice and guinea pigs is that oestrus cannot be induced in guinea pigs since it has a complete oestrus cycle with an active luteal phase. Therefore no mating or stimulation is needed for corpus luteum (CL) production, which meant that the guinea pigs had to be monitored every day to study the different stages of the oestrus cycle using vaginal smear tests and detection of an open vaginal membrane (also unique in the guinea pig). The embryos collected were then incubated at a temperature of 37°C in a concentration of 5% carbon dioxide to study the maturation of the pre-implantation embryos in a medium containing foetal bovine serum (Suzuki *et al*., 2000).

In preparation of this experiment, I first studied and understood the protocol of superovulation used on mice (Nagy *et al*., 2003) by inducing superovulation using equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) and collecting oocytes the day after ovulation took place. This crucial practice and 'The Laboratory Guinea Pig' (Terrill and Clemons, 1998) gave me the confidence needed to work with guinea pigs since this species is not used at Szent Itvan University or any other laboratories in Hungary.

2. Literature Review

2.1 Guinea Pig VS Mouse

The guinea pig (*Cavia porcellus*) is a rodent that in many ways defers from the laboratory mouse (derived from the house mouse *Mus musculus*). Some physical differences are noticed on sight such as the absence of a tail in the guinea pig and the huge difference in size; but more important are the differences in the features related to reproduction as illustrated in the table below:

Table 1. The difference in reproductive features between the guinea pig and the mouse; the data was obtained from 'The Laboratory Guinea Pig' (Terrill and Clemons, 1998) and 'The Laboratory Mouse' (Schweibert, 2007).

	Guinea Pig (*Cavia porcellus*)	**Mouse (*Mus musculus*)**
Weight (g)	600-1000	20-40
Average lifespan (y)	5	2-3
Oestrous cycle (d)	15-19	5
Gestation period (d)	67-69	19-21
Average litter size	3 (1-4)	10 (1-13)
New-borns	precocial	atricial
Vaginal closure membrane	present	absent
Ovarian bursa	absent	present
Active luteal phase	present	absent
Age of puberty (d)	60-90	28-49

There are many advantages of using mice as research animals and their use has resulted in many scientific advancements and biological discoveries. The large number of strains available and different genetic characterisations made them suitable for many different areas of research. Mice are easy to care for and inexpensive compared to other species. They have a high reproductive performance due to their short gestation period and large litter size. This

means that many generations can be reproduced in a short period of time and large pools of data can be obtained. The disadvantages include their small size which limits the procedures that can be performed on the mouse and the possibility of large margins of errors.

On the other hand guinea pigs are large yet still easy to handle due to their good nature. But they have a huge disadvantage as they have a long gestation period and a small litter size. Despite this, the guinea pig is the preferred research animal for the study of reproduction in humans, having many reproductive features similar to those of primates and humans. These include long gestation period, small litter, non-seasonal polyoestrous, spontaneous ovulation, active corpora lutea, complete follicular and luteal phases and fertile post-partum oestrous. The placenta of the guinea pig also resembles that of the human, being a haemochorial placenta.

The boar's (female guinea pig) milk differs from other rodents since it lacks short chain fatty acids, contains 4% fat and 8% protein, resembling the milk produced by canines and humans (Terril and Clemons, 1998). Mice will abort their young once the ovaries have been removed, while guinea pigs manage to carry the foetus to term making them valuable to the study of endocrine control of human pregnancy (Terri and Clemons, 1998). Both the guinea pig and human blastocysts undergo the same interstitial-type implantation and produce syncytiotrophoblasts (Suzuki *et al*. 2003). These features, amongst others, make the guinea pig a better model for the study of human reproduction than mice.

2.2 Superovulation in mice:

For experiments that require a large number of pre-implantation embryos, gonadotropins are often prepared and given to the females prior to mating to increase the number of oocytes ovulated i.e. inducing superovulation. Due to this procedure one may expect about 20 mature ova per female. Pregnant mare serum gonadotropin/Equine chorionic gonadotropin (PMSG/eCG) is used to mimic follicle stimulating hormone (FSH), while human chorionic gonadotropin (hCG) is used to mimic luteinizing hormone (LH) (Coello *et al*., 2008).

The sexual maturity of the female mouse is a major factor affecting the number of eggs that can be produced by ovarian stimulation (superovulation). The best age is 6-16 weeks old, although it is not always a reliable indicator of sexual maturity, as the nutritional status and

health of the female can also affect follicular maturation. Underweight or underdeveloped females produce a lower number of oocytes, even after superovulation. After 4 months of age, the older the mouse is, the lower the egg count.

Ideal females for experimentation should be around 45 days old and weigh between 12 and 14g. The manual: 'Manipulating the Mouse Embryo: A Laboratory Manual' (Nagy *et al,* 2003) recommended a dose of 5IU of eCG. hCG is the second gonadotropin that is administered to induce the final maturation of the oocytes/follicles and ovulation. This serves as a substitute for LH and is required for the matured follicles to rupture.

The times that the eCG and hCG are administered should be relative to each other and to the light-dark cycle of the mouse room. This affects the uniform development and the numbers of eggs that are recovered from superovulated female mouse. There should be a 48-hour interval between the eCG injection and hCG injection as, according to previous studies, this is optimal for high yields of ova. Generally ovulation takes place between 10 and 13 hours after injection of hCG. The time that the endogenous LH is released in response to PMSG is regulated by the light-dark cycle; therefore a cycle of 12 hours of light followed by 12 hours of darkness is applied.

Nine hours after hCG administration, the mice are culled by carbon dioxide gassing and the ovaries, oviducts and uterine horns are harvested and put into the medium. The oviducts are excised and the cumulus-enclosed ova are recovered and put into another petri dish where hyaluronidase containing medium is added. The ova are removed from the cumulus cells by gentle agitation using the micropipette and placed into fresh medium where the numbers of mature ova are determined under the microscope. Mature ova are determined by the presence of a polar body. Immature ova are determined by the presence of a germinal vesicle which makes the ova look fragmented.

2.3 Superovulation Induction in guinea pigs:

It has been proven that eCG and hCG, which are often used in other laboratory animals such as mice, do not work in guinea pigs (Donovan and Lockhart, 1974). This was an incentive for scientists to find the best way to induce superovulation in guinea pigs so as to obtain an

acceptable number of pre-implantation embryos to use in important studies where the guinea pig embryo is the most reliable model.

2.3.1 Using Luteinising Hormone (LH):

Garza *et al.* (1984) attempted inducing superovulation in the hamster using osmotic mini-pumps containing 400µg of ovine LH installed subcutaneously on day 1 post ovulation. The results showed that this method increased the number of oocytes. Using a similar method he tested this protocol on other rodents including rats and guinea pigs. The application of LH on day 12 post ovulation in the guinea pig doubled the number of oocytes ovulated. The results showed that the exogenous LH might induce superovulation by preventing atresia of the developing follicles.

2.3.2 Using pregnant mare's serum gonadotropin (PMSG):

Kosaka and Takahashi (1989) investigated the ability of PMSG to induce superovulation on guinea pigs with synchronised oestrus cycles which were regulated using progesterone tubing for 21 days. 6 days after ceasing the progesterone treatment, every guinea pig given a saline injection had synchronised ovulation. The guinea pigs injected with PMSG 8 hours before the removal of the progesterone tubing, had a higher rate of ovulation. There was no increase of oocytes ovulated with any other treatment schedule. This study indicated that PMSG treatment was successful at inducing superovulation, but it is also time-dependent and guinea pig's eostrous cycle should be synchronised using progesterone tubing for a relatively long time.

2.3.3 Using inhibin vaccine:

Shi *et al.* (2000) carried out an experiment to find out whether neutralising inhibin would affect follicular development and ovulation rate in guinea pigs. They used 3 groups of guinea pigs, all of which had a progesterone implant for 4 weeks. Group 1 was given a placebo consisting of 1ml saline in oil emulsion; group 2 was given 25µg of inhibin vaccine while group 3 was given 50µg of inhibin vaccine; 3 times at 4 week intervals. Blood samples were collected from each group once a week to measure inhibin antibody titres. After the 3rd

injection, blood samples and ovaries were collected from the 3 groups on day 8 post-ovulation. The ovaries were fixed in formaldehyde and sectioned (10µg thick) before being prepared for light microscopy and stained with haematoxylin and eosin.

The blood samples collected each week showed an increase in titre of inhibin antibodies after each injection. The follicles that contained a visible oocyte nucleus and some granulose cells were taken into consideration after studying the slides previously prepared. The results showed that the inhibin vaccine increased the rate of ovulation (based on the number of CL found) but it was also dose-dependent, i.e. the higher the dose of inhibin the larger the number of oocytes/CL present (group 1: 4.2± 0.9; group 2: 6.2± 0.9; group 3: 9.8± 0.9).

Active immunisation against a recombinant ovine α-subunit results in the production of circulating anti-bodies that are able to bind to the inhibin produced by the guinea pig. These anti-bodies neutralise the negative feedback given by the endogenous inhibin on the secretion of FSH by the pituitary gland to stimulate follicular development and in return, induce superovulation.

2.3.4 Using human menopausal gonadotropin based on follicular waves and FSH-R homologies:

Suzuki *et al*. (2003) studied the availability of ovarian oocytes during the oestrous cycle and follicular stimulation hormone receptor (FSH-R) homologies between guinea pigs and other species to identify an effective gonadotropin and optimal time of application for a successful superovulation. The deduced amino acid sequence of guinea-pig FSH-R proved to be similar to the primate's sequence rather than other rodents', therefore hMG was applied for induction of superovulation. The cDNA sequence of the FSH-R was deduced by a combination of 5'- and 3'- rapid amplification of cDNA ends (RACE) together with an adaptor-ligated, double stranded cDNA library.

Just as most domestic animals have species specific waves of follicular growth, such as the cow and sheep, the guinea pig also has more than 1 follicular wave during an oestrous cyclic, when a large number of oocytes are available. Therefore they used two groups of guinea pigs: one group was induced with hMG during the mid- luteal phase and the other group was induced during the pre-ovulatory phase. 5IU of hMG was applied on three consecutive days

during the afore-mentioned phases. When guinea pigs were allowed to ovulate spontaneously, without the help of gonadotropins, the mean number of oocytes detected by the number of corpora lutea (CL) present on the ovary post ovulation was 3.6 ± 0.1, (O.Suzuki *et al.* 1993). The guinea pigs which were induced mid-luteal phase did not ovulate at all, despite having a good number of oocytes available. This could be due to the high level of plasma concentration progesterone secreted from active CL. This makes the guinea pig suitable to evaluate the affects of CL on induction of ovulation in humans. The guinea pigs induced just before ovulation showed an increase of oocytes: 5.4 ± 1.6, range = 0-17. This proved that hMG was effective but also stage-dependent.

Gregoire *et al*. (2011) used hMG to induce superovulation in guinea pigs with open vaginal membranes. An average of 4.75 embryos per guinea pig were flushed at morula and early blastocyst stage on day 3.5 and 4.5 post ovulation and were then transferred to synchronised females on days 3.5 and 4.5 post ovulation. Two embryos where inserted into each uterine horn using pulled glass pipette or a plastic open pulled straws. One of the surrogate guinea pigs impregnated using a pulled glass pipette gave birth to two stillborn pups, one from each uterine horn. Further studies should be made to improve this method in order to achieve a successful protocol for embryo transfer.

2.4 Maturation of Pre-implantation Embryos in vitro:

After using inhibin vaccine to induce superovulation, Shi *et al*. (2000 b) discovered that there were no harmful effects on the embryo quality and its development. By comparing the results to those of a control group they realised that embryos flushed on day 6 of pregnancy reached the blastocyst stage with little fragmentation. The blastocysts also hatched and formed trophoblasts during *in vitro* culture using a mixture of RPMI 1640 and Dulbecco's Modified Eagle's Medium containing 0.3% bovine serum albumin, proving that this medium is ideal for maintaining the growth of pre and peri- implantation embryos collected on day 6 of pregnancy. Studies on the use of this medium on embryos at earlier stages before day 3 of pregnancy have yet to be made, especially since most embryos arrested their development at the 4 cell stage when using Kane's or Whitten's medium (Suzuki *et al*., 1993). This data supports the use of inhibin vaccine as a safe method of inducing superovulation in guinea pigs.

3 Materials and Method

3.1 Animals and Husbandry:

The guinea pigs used were common pet guinea pigs of different breeds such as Common Short haired, Abyssian and Sheltie (Figure 1 a, b and c respectively). They were bought randomly from breeders at 2-6 months and kept in wire-topped cages using corn cob litter as bedding. They were fed a diet of fresh produce rich in vitamin C, commercial food for guinea pigs, hay and water *ad libitum*. They were housed in a room with a constant temperature of 20 ± 1˚C and at least 8 hours of light during the day.

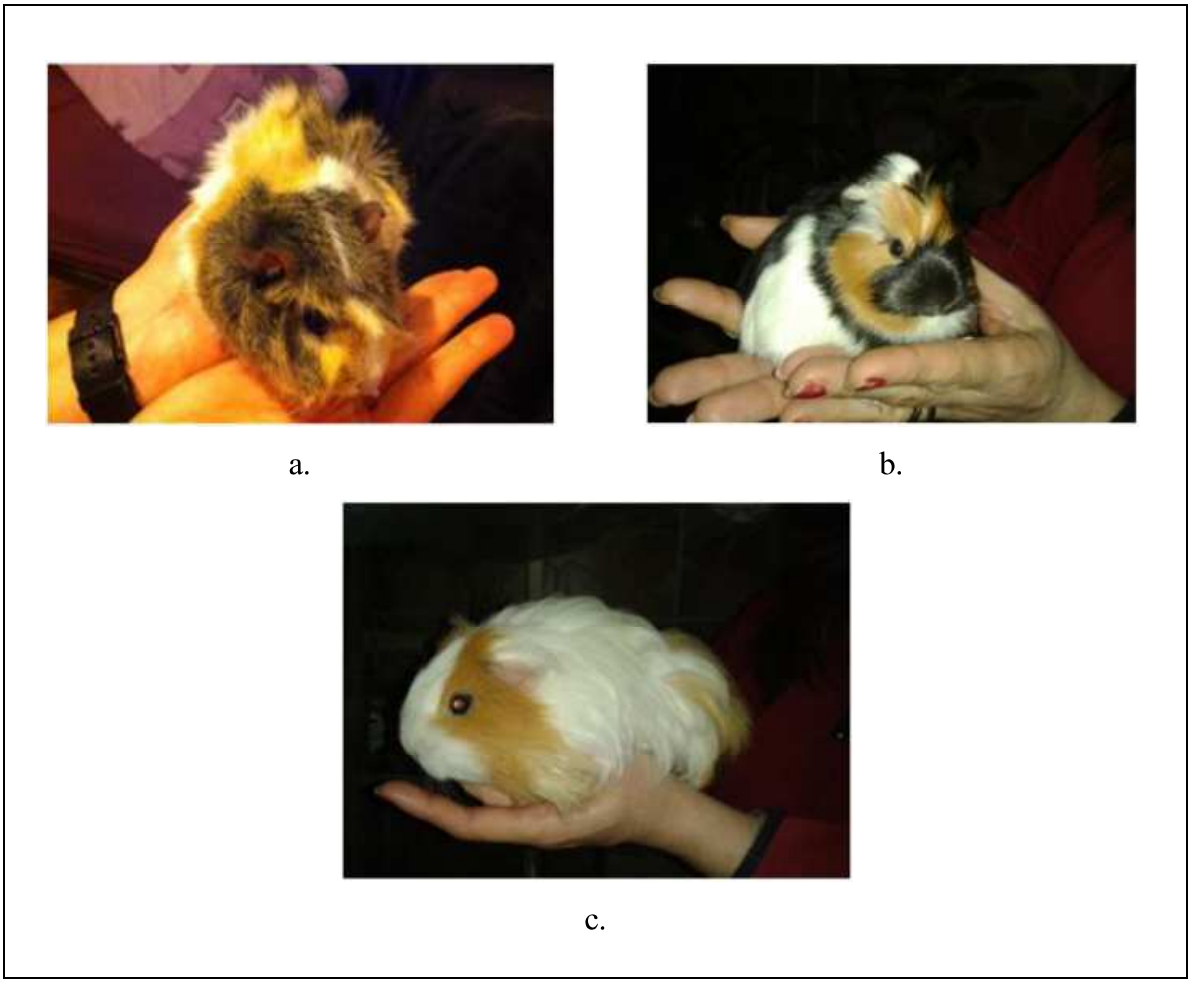

Figure 1. Abyssian guinea pig (a.), common short-haired guinea pig (b.), Sheltie guinea pig (c.)

It was important that the female guinea pigs were at least 2 months of age when they were bought and were observed for 6-8 weeks to make sure that they ovulate regularly. This was done by taking vaginal swabs every morning between 7:00 and 8:00, using cotton tips soaked in saline solution while the vaginal membrane was open. The vaginal smears were checked under the microscope and assessed based on the cells present. The behavioural changes such as strutting, purring and chasing other female guinea pigs were also indicative, but not precise.

3.2 Inducing Superovulation:

Once a regular cycle is confirmed, the days on which the hMG was to be administered were decided. The hormone was given on days 12, 13 and 14 post ovulation which was a modification made to Suzuki *et al.* (2000)' s protocol since the oestrus cycle was relatively shorter (15-17 days) compared to that of the guinea pigs Suzuki *et al*. (1993) used: 20-25 days.

The guinea pig was turned on its back and held steadily using one hand while administering 5cc of hMG intraperitonealy using a 16 gauge, 0.5mm hypodermic needle and a 1 ml sterile syringe. Care was taken to avoid hitting the diaphragm and the bladder (Figure 3). In the meantime vaginal smears were taken while the vaginal membrane was open (Figure 2) and when a large amount of cornified cells were present (Figure 10) the boar was introduced to the stud in his cage. Once copulation took place a vaginal plug (seminal fluid) was noticed and this was confirmed by another vaginal smear which showed the presence of spermatozoa and leukocytes (Figure 11).

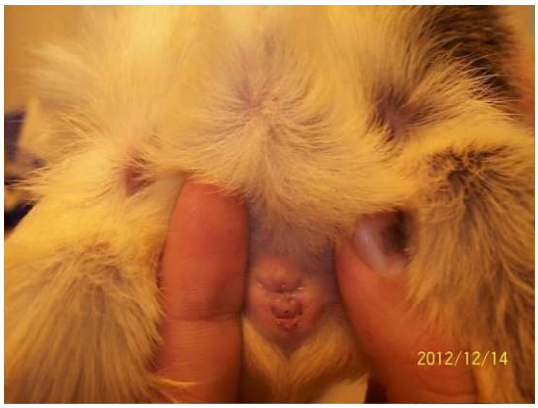

Figure 2. A ruptured vaginal membrane during oestrous.

Figure 3. The guinea pig was turned on its back while 5IU of hMG were administered intraperitoneally.

3.3 Retrieving the uterus by ovario-hysterectomy:

The embryos were collected on day 4 post-coitus, following Suzuki *et al*. (2000)'s protocol. To avoid culling the guinea pigs, an ovario-hysterectomy was performed to retrieve the uterus. The surgical procedure was adapted from the book 'Ferrets, Rabbits ad Rodents: Clinical Medicine and Surgery' (Carpenter and Quesenberry, 2011). The guinea pig was anaesthetised using the appropriate amount of xylazine and ketamine, which was calculated based on its weight. The abdomen was clipped and prepared for aseptic surgery while placed in dorsal recumbence and then draped appropriately. A heat pad was placed under the guinea pig to prevent hypothermia and an anaesthetic machine was used in order to maintain oxygen flow and narcosis with isoflurane gas by inhalation (Figure 4).

Figure 4. The guinea pig was anaesthetized and prepared for aseptic surgery.

A 4cm incision was made using the umbilicus as the centre. Care was taken to avoid iatrogenic injury to the caecum and bladder. The uterus was located between the bladder and the colon and the left horn was followed cranially to locate the ovary behind the left kidney. The vessels supplying the ovary within the mesovarium were identified, and using blunt dissection, an opening was created at the border of the mesovarium and mesometrium to allow placement of the two consequent ligatures of an absorbable synthetic suture material. The suspensory ligament, mesovarium and the vessels distant to the ligature were transected. The procedure was repeated on the contra lateral side and the broad ligament on each side of the uterine body was bluntly dissected. The uterus was ligated cranial to the cervix. This together with the ovaries were removed and placed in a container filled with bovine serum and phosphate buffered saline solution and kept at a temperature of at least 35°C, until it was transferred to the laboratory. The abdomen was closed with a 4.0 monofilament absorbable suture material using a simple continuous pattern for the subcutaneous layer and intradermal continuous pattern to finish off.

3.4 Preparing the mediums for the embryos:

The medium used is Sydney IVF Cleavage Medium K-SICM-100. This medium is ideal to provide the necessary nutrients for human embryo development in vitro, but it is also suitable

for mouse and guinea pig embryos. This medium is supplemented with Human serum albumin (5mg/ml) and gentamycin (0.01 mg/ml).

The culture medium was prepared in a 4-well plate a day before the collection of embryos. 400µl of Cleavage Medium was placed into each well and 400µl of mineral oil was added by placing the micropipette on the walls of the wells. The purpose of the culture oil is to prevent evaporation of the medium and in turn, desiccation of the embryos. The 4-well plate was partially covered with the lid before being placed in the thermostat for one day, set at 37.5˚C in an environment of 6.5% carbon dioxide in the air for equilibration. This Medium is ideal for the growth of embryos to blastocyst stage.

The medium used for recovering the embryos under microscope was also prepared beforehand. Using a 5 ml pipette, 8ml of Dulbecco's phosphate buffered saline and 2ml of bovine serum was placed into 4 test tubes, which were then homogenised. A 10ml syringe was filled with the homogenised mixture and the needle was replaced with a filter. The bottom of five 35mm petri dishes was covered with the medium and was later placed on a hot plate, equilibrated at 37°C (Figure 5).

Figure 5. Petri dishes containing homogenised medium on a hot plate at 37 ˚C

3.5 Flushing the uterus and collecting the embryos:

Using the stereomicroscope for higher magnification, the ovaries and fat were removed and the uterine body and horns were transferred into a fresh petri dish previously prepared. A hypodermic needle and syringe containing some of the medium was used to flush out the embryos. One of the uterine horns was removed at the bifurcation using fine scissors and flushed by injecting the medium at the end of the horn (where the oviduct was connected), allowing the embryos to flow out of the end where the uterine horn was severed. The procedure was repeated with the other horn and the body was also flushed as a precaution. The remains of the uterus were discarded appropriately.

Using the stereoscope, all the viable embryos were picked up using a micropipette and placed in the previously prepared 4-well plate (Figure 6).

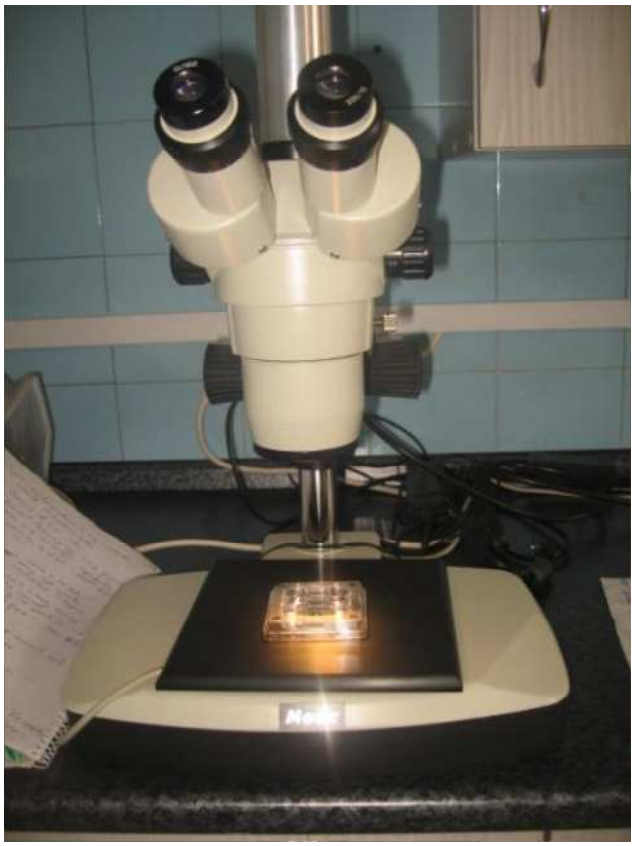

Figure 6. The embryos were transferred to a 4-well plate containing Cleavage Medium

3.6 In vitro Culture of the Pre-implantation Embryos:

The pre-implantation embryos were collected on day 4 post-copulation and were expected to be 4 or 8-cell stage zygotes. The embryos were transferred to the 4-well plate and the well used was labelled with the date and number of the guinea pig the embryos were collected from. The 4-well plate was then covered with the lid and placed in the incubator at and stored 37.5˚C and 6.5% carbon dioxide concentration to allow the embryos to grow at optimal conditions. The embryos were taken out of the incubator everyday for up to 5 days, between 14:00 and 15:00 to study the growth until the embryos become blastocysts or collapse.

4 Results

4.1 Induction of Superovulation:

The most important step of the method used was determining the length of the oestrus cycle in each guinea pig. This ensured that the hMG were administered during the pre-ovulatory phase. Suzuki *et al.* (1993)'s results show that hMG is time-dependent since no oocytes were ovulated when the hMG was administered during the luteal phase after the first follicular wave of the oestrus cycle. Vaginal smears were prepared for viewing under the microscope while the guinea pig was in heat and the vaginal closure membrane was open.

The guinea pig's vaginal membrane was checked every morning. On the same day that the vaginal membrane had ruptured the guinea pig also showed behavioural changes including swaying, purring and courting other cage mates. This generally occurs about 50 hours prior to oestrus as recorded in 'The Laboratory Guinea Pig' (Terrill and Clemons, 1998). The vaginal smear prepared that day showed the presence of parabasal and intermediate epithelial cells with large nuclei (Figure 7). On the next day, the epithelial cells had started to acquire rough edges and the nuclei started to shrink (Figure 8). This gradual shift from parabasal and intermediate epithelial cells to polygonal superficial cells shows that the guinea was in proestrus. Some leukocytes also appeared in small amounts although they are of little significance at this stage (Figure 9).

By the 3rd day since the opening of the vaginal membrane, the epithelial cells became polygonal and the nuclei disappeared; these are called cornified cells which are usually found in clumps (Figure 10). This meant that the guinea pig was in oestrus but ovulation had not occurred yet. While in oestrus the guinea pig was seen exhibiting copulatory reflex; the guinea pig was lowering its back while raising its posterior, accepting the male for coitus. Ovulation usually occurs around 10 hours after onset of oestrus and this was confirmed by the presence of a large number of leukocytes seen under the microscope when a vaginal smear was prepared the following morning. The vaginal membrane closed again about 24 hours after ovulation took place. The time between one confirmed ovulation and the next was considered

to be the length of the oestrus cycle but at least 3 oestrus cycles were observed to make sure that the oestrus cycle was regular.

Once the guinea pig became suitable for superovulation, hMG was administered on day 12, 13 and 14 before the boar was placed in a cage with a mature male, so as to allow copulation while in oestrus. A copulatory plug was sometimes found after copulation, which fell out only a few hours later. In some cases the copulatory was not seen and so copulation was always confirmed by preparing a vaginal smear the morning after ovulation took place. The slide showcased a large number of spermatozoa and leukocytes and so this was marked as day 1 post coitum (p.c.) (Figure 12).

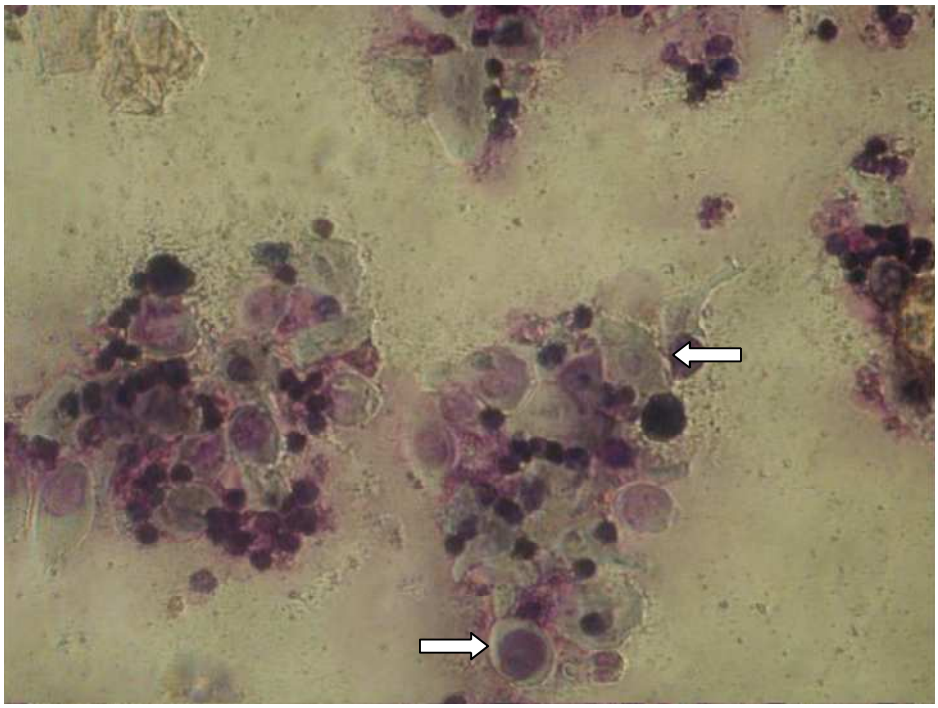

Figure 7. Vaginal smear showing parabasal cells and intermediate epithelial cells x400

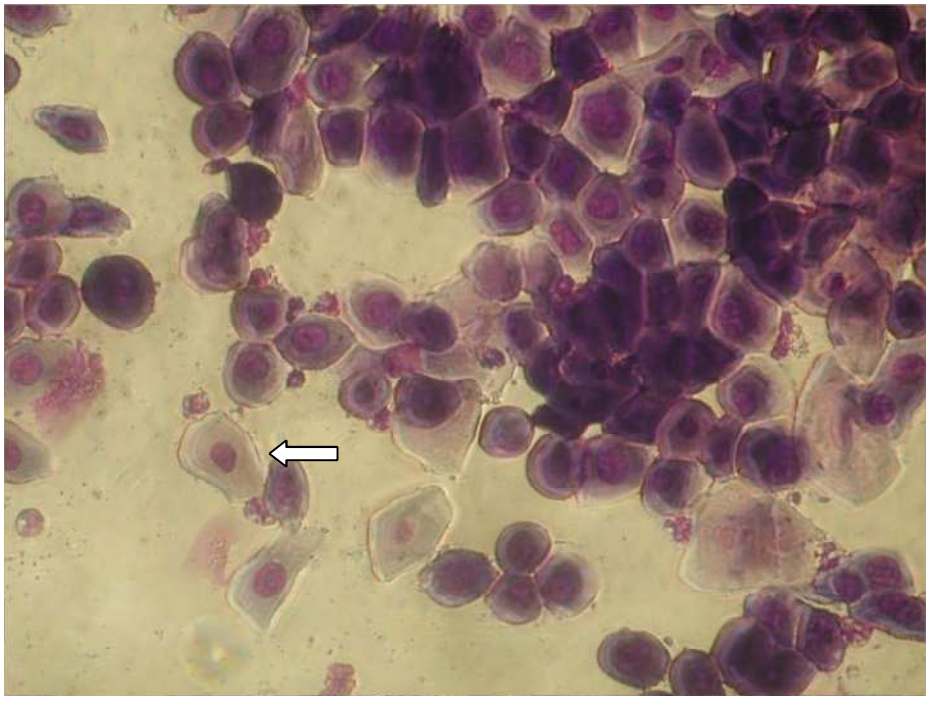

Figure 8. Vaginal smear showing polygonal epithelial cells with smaller nuclei x400

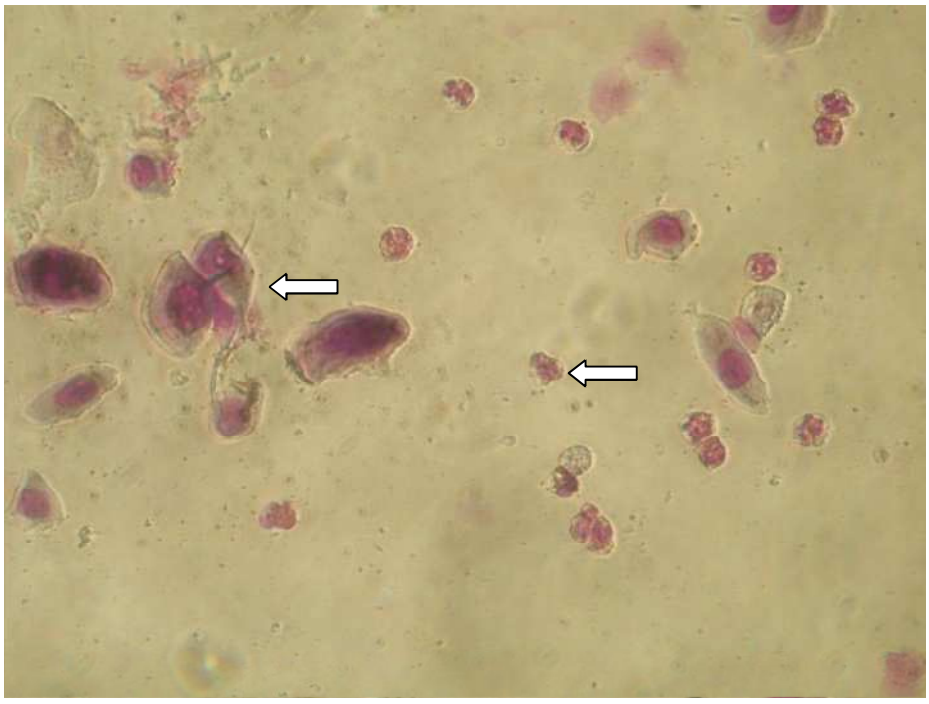

Figure 9. Vaginal smear showing surperficial epithelial cells and leukocytes x400

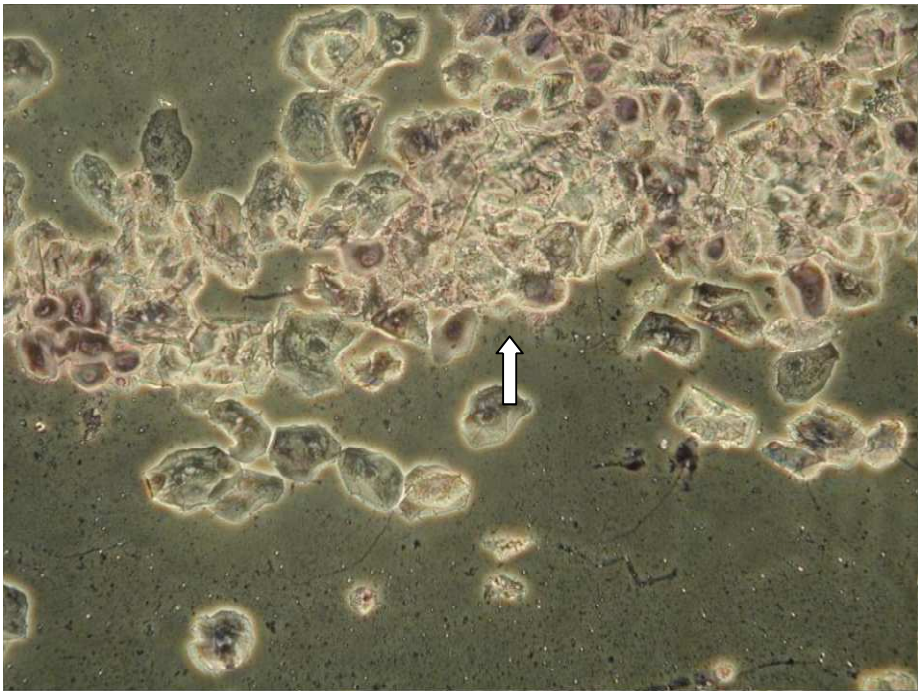

Figure 10. Vaginal smear showing cornified cells in clumps x400

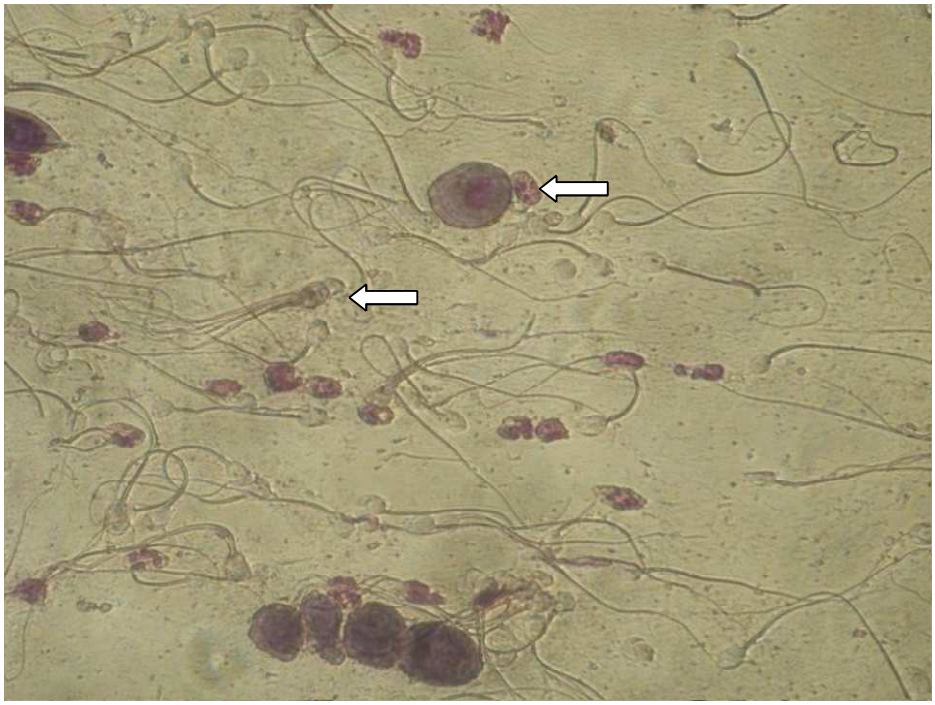

Figure 11. Vaginal smear showing spermatozoa and epithelial cells x400

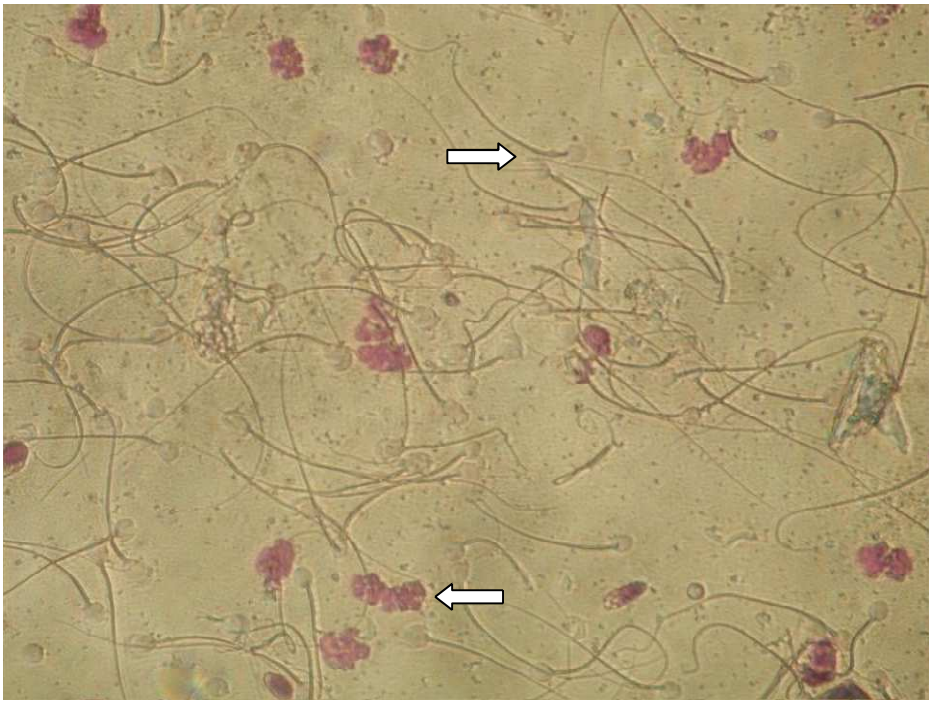

Figure 12. Vaginal smear showing spermatozoa and leukocytes x400

4.2 Embryo Collection and Development in vitro

On day 4 p.c., an ovario-hysterectomy was performed on the guinea pig and the embryos were flushed from the uterus. The guinea pigs which went through this procedure (guinea pigs 1, 2, 3, 6 and 7) ovulated at least 4-7 oocytes; giving an average of 5.2 embryos collected per guinea pig. It is probable that more oocytes were ovulated but were not collected due to early implantation, amongst other reasons. This could have been compensated by preparing microscopic slides of the ovaries and counting the CL, as was done by Suzuki *et al.* (2003) and others. When oocytes were collected from mice which had superovulated, the number of oocytes was compared to the number of corpora haemorrhagica which could be seen under the microscope. This method was not suitable for the guinea pigs since the embryos were collected 4 days p.c. by when the corpora haemorrhagica would have turned into CL.

Another peculiar finding was that the embryos collected from guinea pig 1 and 6 seemed to come from one uterine horn and only 1 embryo was collected from the contra lateral uterine horn in guinea pig 2. This also showed the possibility that the number of oocytes ovulated could have been larger than the number of embryos collected.

Table 2. The age of each donor guinea pig, the number of embryos collected from each guinea pig, the total of embryos flushed and the average of embryos calculated.

	Age (months)	**Embryos Collected**
Guinea Pig 1	5	4
Guinea Pig 2	4.5	4
Guinea Pig 3	7	6
Guinea Pig 6	5	5
Guinea Pig 7	5.5	7
Total	-	26
Average	-	5.2

The embryos of guinea pig 1, 2 and 3 were incubated at a constant temperature of 36.5ºC and a concentration of 6.5% carbon dioxide for 5 days during which their development was observed and recorded. Most embryos were at an 8-cell stage or 16-cell stage when flushed on day 4 of pregnancy. 50 % of the embryos collected from guinea pig one and 75% of the embryos collected from guinea pig 1 and 2 respectively, developed into blastocyts.

Table 3. The stages of embryo development *in vitro*. Day 1 was the day the embryos were flushed and collected.

	Guinea pig 1	Guinea pig 2	Guinea pig 3	Guinea pig 6	Guinea pig 7
Day 1	8-cell morula	8/16- cell morula	16-cell morula	8-cell morula	8-cell morula
Day 2	16-cell morula	32- cell morula	32-cell morula	-	-
Day 3	32-cell morula	32- cell morula	-contaminated	-	-
Day 4	blastocyst	blastocyst	-	-	-
Day 5	blastocyst	blastocyst	-	-	-

The following photos show the progression of the embryos' development into blastocysts, except for the embryos of guinea pig 3 whose development was ceased after fungal contamination. The embryos of guinea pig 6 and 7 were not incubated since the necessary equipment was not available at the time of the flushing.

Guinea pig 1:

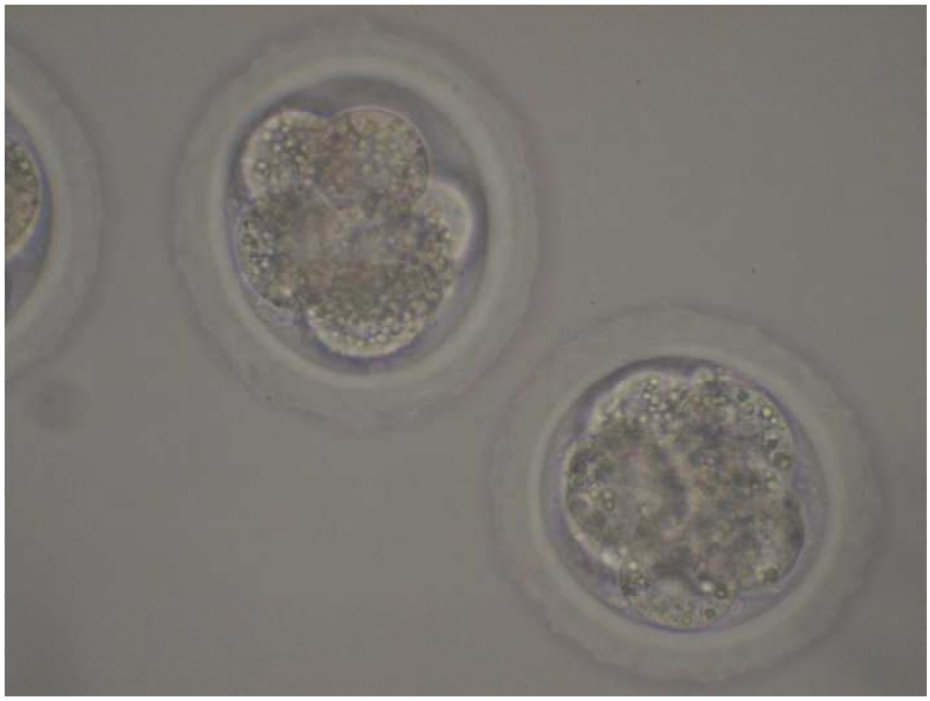

Figure 13. Day 1: 8 cell morula x400

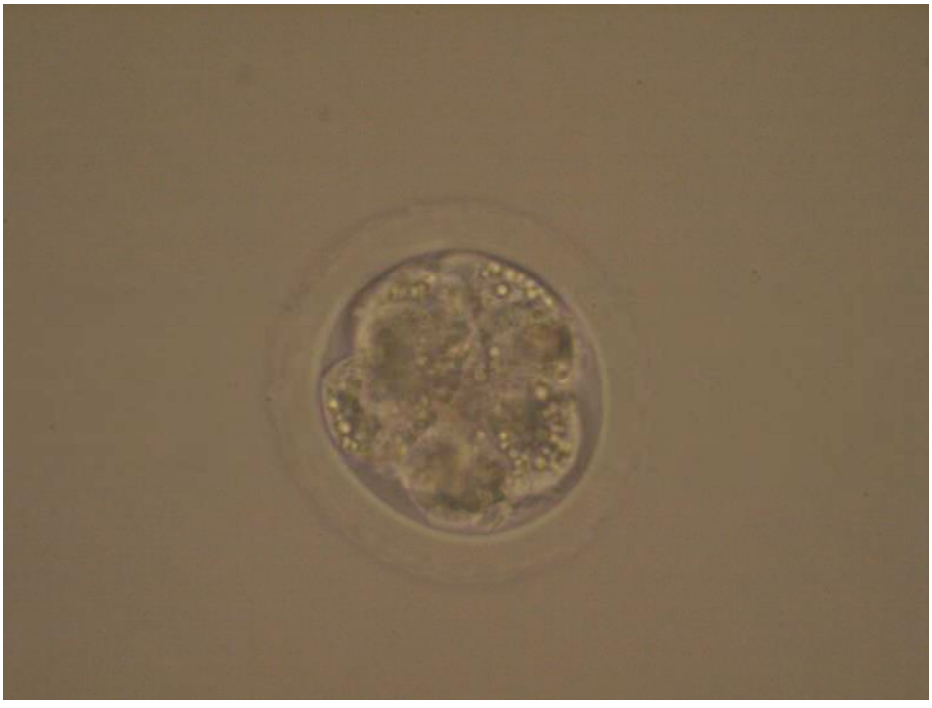

Figure 14. Day 2: 16 cell morula x400

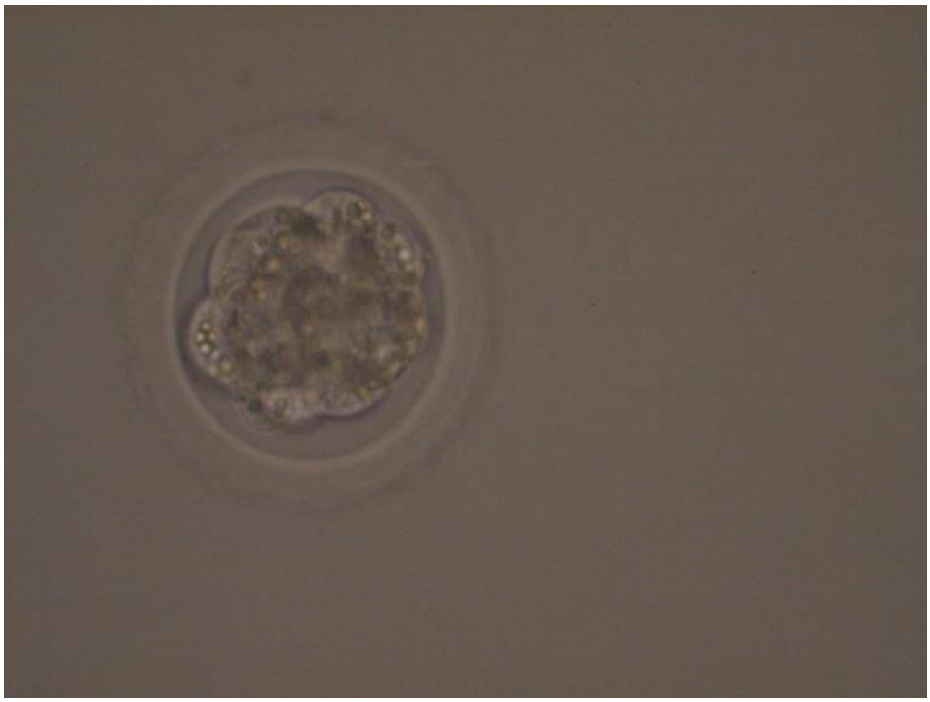

Figure 15. Day 3: 32 cell morula x400

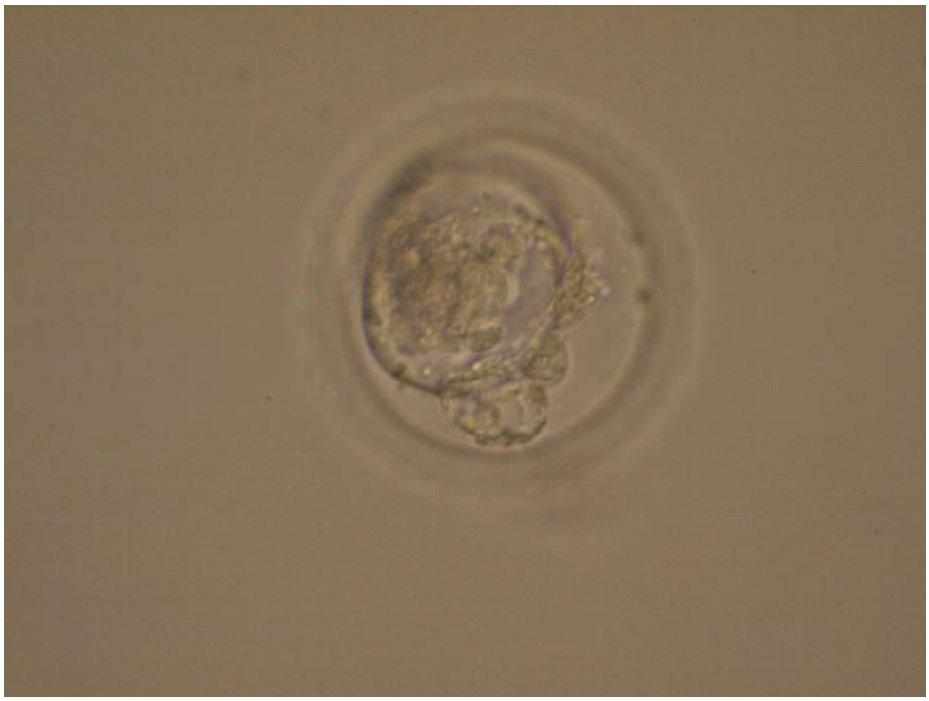

Figure 16. Day 4: blastocyst x400

Guinea pig 2:

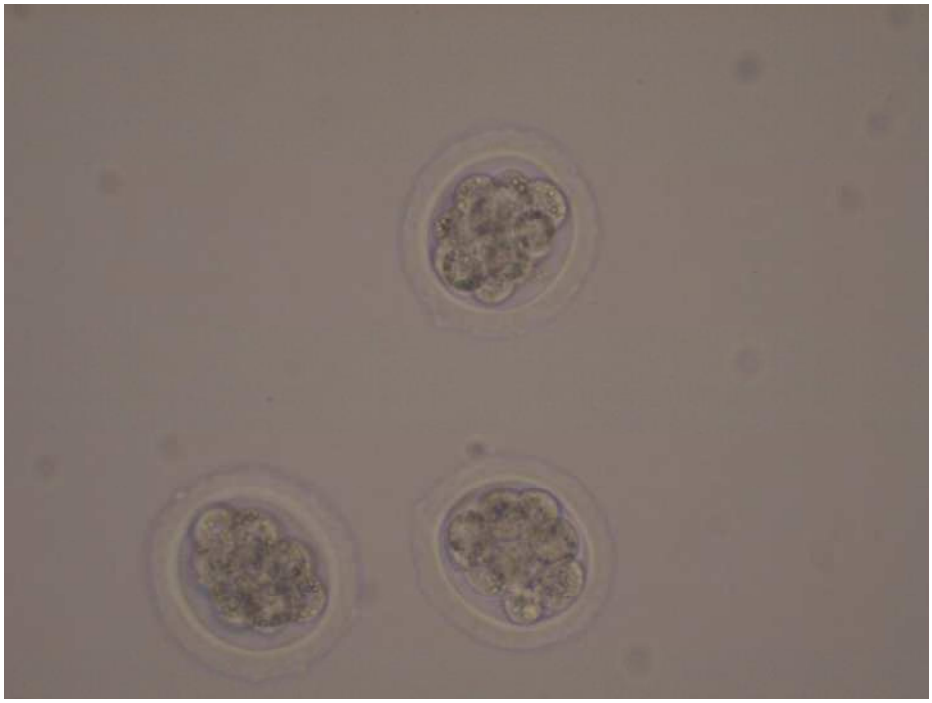

Figure 17. Day 1: 16 cell morula x400

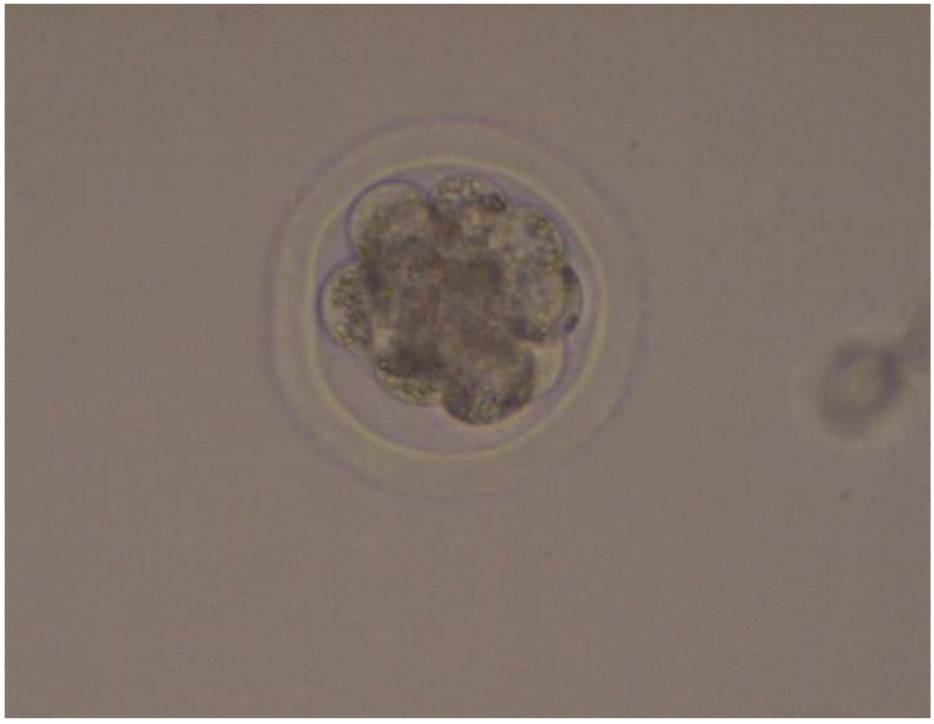

Figure 18. Day 3: 32 cell morula x400

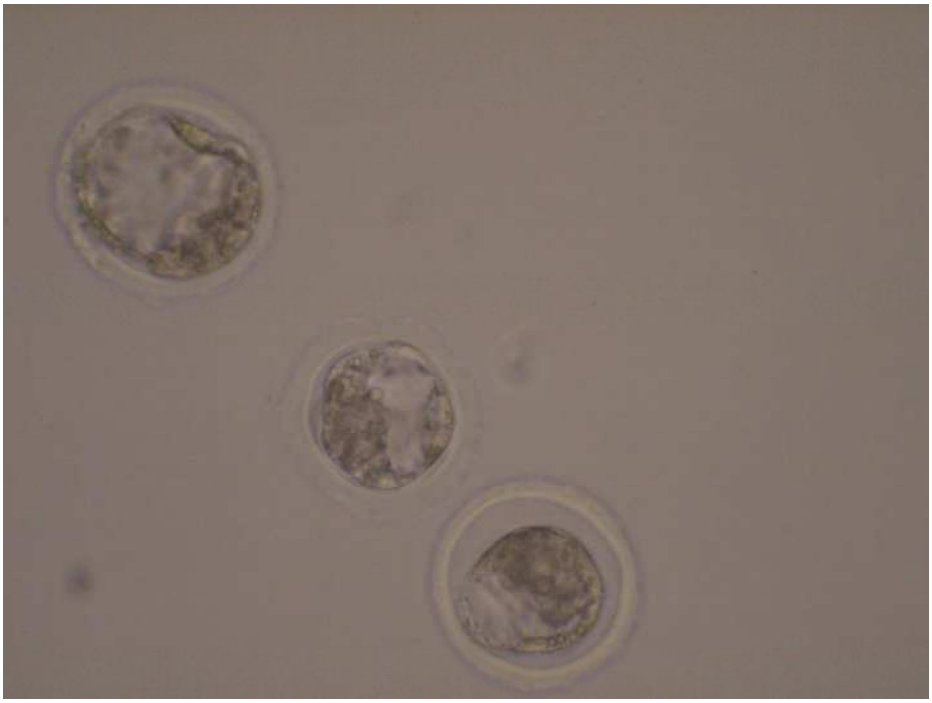

Figure 19. Day 5: blastocysts x400

Guinea pig 3:

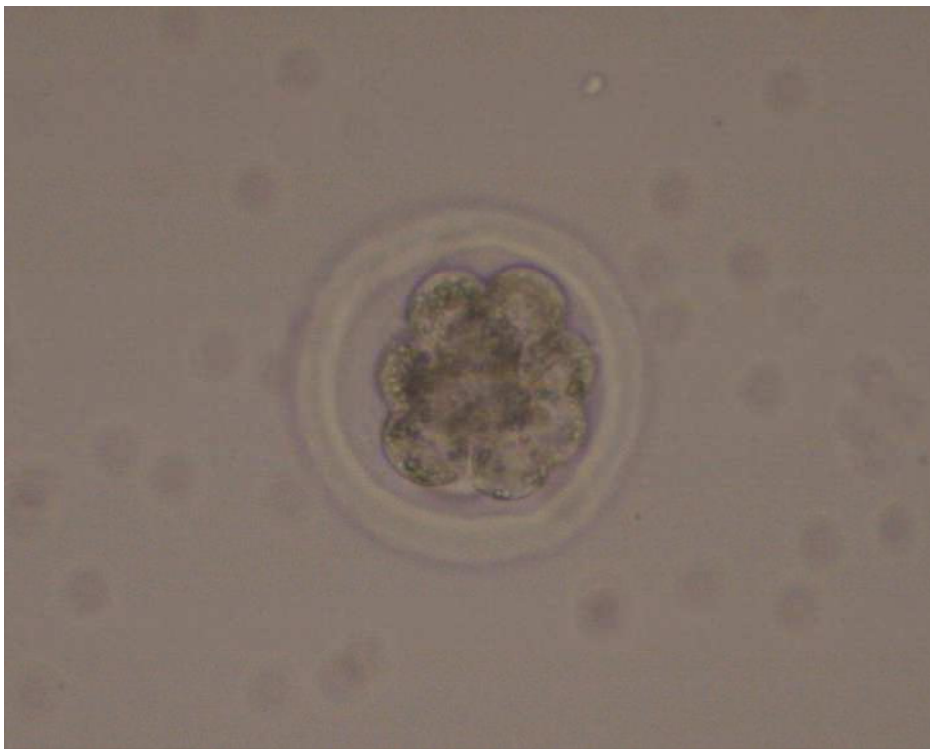

Figure 20. Day 1: 16 cell morula x400

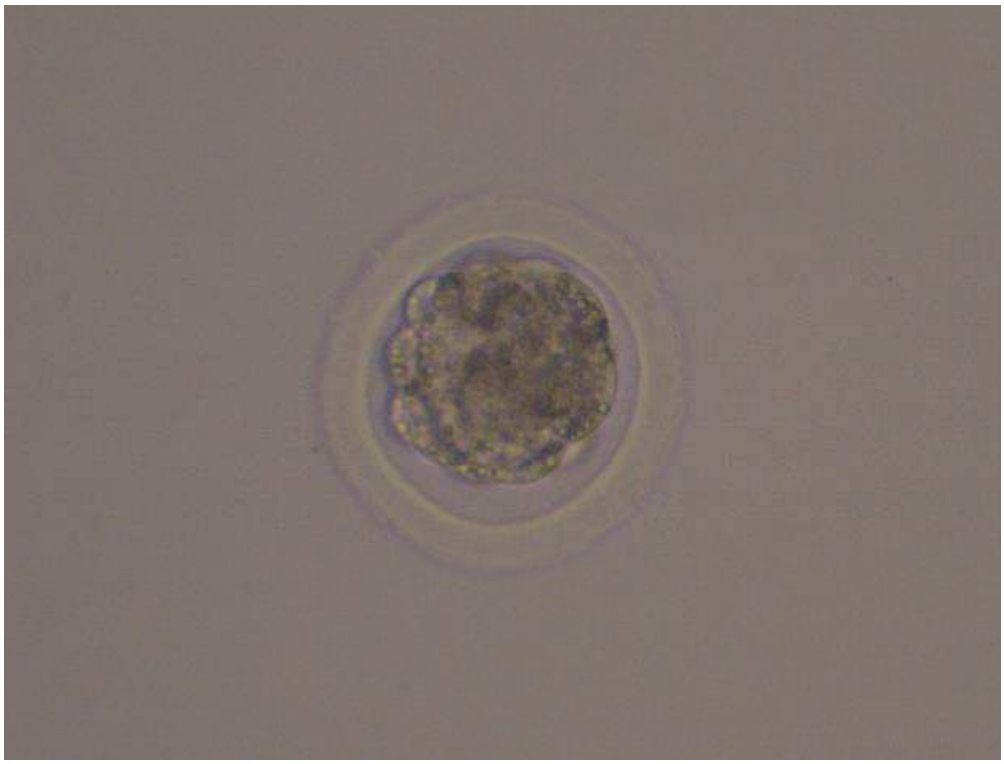

Figure 21. Day 2: 32 cell morula x400

5 Discussion

5.1 The oestrus cycle of the guinea pig:

The guinea pigs have a complete oestrus cycle that is generally 15-19 days long (Terril and Clemons, 1998) but according to O.Suzuki (personal communication), the oestrus cycle of the Hartley strain is 20-25 days long. To find out the length of oestrus cycle of the pet guinea pigs used for this experiment, the behaviour of the animals were observed for 6-8 weeks and smears of the vagina were studied under the microscope while the vaginal closure membrane was open.

Although they are not reliable on their own, the behavioural changes can determine the stage of the oestrus cycle the guinea pig is in. When the female starts courting other females by swaying, purring and chasing them, the guinea pig is said to be in proestrus. This starts 50 hours before oestrus and towards the end of it the vaginal membrane ruptures. While in oestrus the female exhibits copulatory reflex which includes lordosis and elevating the posterior. Ovulation occurs 10 hours after onset of oestrus (Terril and Clemons, 1998).

The male was added towards the end of proestrus and copulation occurred during oestrus when the female accepted him. A copulatory plug was sometimes found, confirming that copulation happened a few hours before. This plug is made of fluid from the seminal vesicles and the prostate glands of the male which coagulates immediately after coitus and falls off a few hours later.

During proestrus the vaginal smears would only show parabasal and round epithelial cells. When the guinea pig was in oestrus a large number of cornified cells appeared but only a few or no leukocytes were present. If a high number of leukocytes were present it meant that the guinea pig ovulated the night before; but if spermatozoa are also present, copulation occurred during the previous night. The data collected showed that the length of the oestrus cycle of the pet guinea pigs used for this experiment ranged from 15 to 17 days and therefore days 12, 13 and 14 were chosen for hMG treatment.

Guinea pigs 4 and 5were bought at 2 months of age and they started ovulating right away. Since they were born and raised together they also happened to have a synchronised cycle which made it easier to work with. The vaginal smears recovered from them gave very clear slides. They also happened to be almost synchronised (just a day apart) since they were born and raised together and their behavioural changes were just as previously explained. During one cycle, an intact layer of cornified cells (Figure 19) came off the vaginal wall when the swab was pulled out of the vagina of guinea pig 4. This has never happened before as usually the layers of dead cells were always recovered in small clumps. No record of an intact layer of cornified cells was found in other research papers and books which raised many questions as to why this happened.

Figure 22. An intact layer of cornified cells was recovered from guinea pig 4; it's an exact mould of the vagina

During the 4th oestrus cycle the 2 guinea pigs were given the hCG but the following vaginal smears showed no sign of ovulation and so they did not undergo ovario- hysterectomy. Despite being in the presence of a male no spermatocytes were found but a high amount of red blood cells and leukocytes were observed on the vaginal smear. The vaginal swabs also showed the presence of brown vaginal discharge (blood and mucous). During the next oestrus cycle the same procedure was applied with the same negative results. This time rod- shaped and coccoid bacteria were found on the vaginal smear which was probably the cause of a

subclinical infection (vaginitis). Therefore the guinea pigs were no longer good candidates for super-ovulation and embryo recovery and were not included in the results.

5.2 Induction of Superovulation

After careful consideration Suzuki *et al*. (2000)'s protocol was followed but some modifications were made to make it suitable for the pet guinea pigs used. Dorsch *et al*. (2008) also used a modified version of this protocol with successful results, which gave us the incentive to use this method. It also appeared to be the most affordable.

Suzuki *et al*. (2000) used hMg during the pre-ovulatory phase to induce superovulation in 'Hartley' laboratory guinea pigs, whose oestrous cycle is generally 20-25 days long (personal communication with O. Suzuki). Therefore hMG was administered on day 14, 15 and 16 of the oestrous cycle. Guinea pigs of inbred strain 2BS was used by Dorsch *et al*. (2008) who have an oestrous cycle length of 17-19 days and so day 14, 15 and 16 were suitable for the gonadotropin hormone. In this experiment, pet guinea pigs of no particular strain were used and after careful observation for 6-8 weeks it was determined that their oestrous cycle length was 15-17 days long which meant that the days mentioned above would have overlapped with ovulation. In this case, days 12, 13 and 14 were chosen for the application of hMG.

The day on which the embryos were to be collected was also taken into consideration. After corresponding with O. Suzuki via email, it was suggested that day 4 would be ideal and this was accepted. Ueda *et al*. (1994) did not recover any embryos from the uterus until day 1 since they were still in the oviduct. Dorsch *et al*. (2008) collected embryos on day 2.5, 4.5 and 6.5 and reported that more embryos were found on day 2.5 than day 4.5, while no embryos were found on day 6.5. From day 6 onwards, the embryos would have been implanted in the epithelium of the uterus.

The exact number of oocytes ovulated was detected based on the number of CL present in the ovaries. This was not known when day 4 was chosen as the day of embryo collection and unfortunately, the equipment necessary for the preparation of microscopic slides of the ovaries was not available. The implications of this error could be that more embryos were probably ovulated than those that were recovered, but the exact number remains unknown.

Therefore the results, 5.2 embryos, are only based on the number of embryos collected from the uterus.

The other reason why day 4 was chosen is that embryos collected at 8 cell stage morula have a better chance of developing into a blastocyst than embryos collected on day 2.5 (Dorsch *et al*. 2008).

Table 2. The results acquired from this experiment compared to the mean number of embryos collected by Suzuki *et al*. (1993, 2003), Dorsch *et al* (2009) and *Gregoire et al*. (2011).

	Days post-coitum (embryo collection)	**Results** (mean no. of embryos)	**Type of ovulation**
O.Suzuki *et al.* (1993)	4	3.6 ± 0.1	spontaneous ovulation
O.Suzuki *et al.* (2003)	4	5.4 ± 1.6	superovulation by hMG
M.M. Dorsch *et al.* (2009)	4.5	5.66 ± 1.99	superovulation by hMG
Gregoire *et a.l* (2011)	3.5/4.5	4.75	Superovulation by hMG
Thesis	4	5.2	superovulation by hMG

Despite the fact that the CL were not counted, the mean number of embryos collected during this experiment was very close to that of Suzuki *et al*. (2003) and Dorsch *et al*. (2009) collected on day 4 and day 4.5 p.c. respectively.

5.3 Development of pre-implantation embryos *in vitro*:

Another particular feature of hMG was observed on more than one guinea pig; when hMG was applied on days 12, 13 and 14 to a guinea pig with a 17 day long oestrus cycle, it was noticed that the vaginal closure membrane opened on day 15, followed by copulation the next night. Hence, hMG can also speed up the growth of oocytes and ovulation if applied during the pre-ovulatory phase. This was also observed by Dorsch *et al*. (2009) who compared the

rate of development of embryos collected from superovulated females to that of untreated females. The rate of development of the embryos collected from this experiment was very close to the rate of that of embryos retrieved from treated females on day 4.5 p.c. by Dorsch *et al*. (2009).

As seen in Table 3, most embryos were 8-cell stage morulae by day 4 p.c. and if given the chance to be incubated for a longer period of time, the embryos had the potential to become blastocysts. In fact 50% of the embryos collected from guinea pig 1 and 75% of the embryos collected from guinea pig 2 reached blastocyst stage, 3 days after collection. Unfortunately the embryos from guinea pig 3 were contaminated by a fungus which was being used by other laboratory technicians in the same lab and so development was ceased at 32-cell stage morula before they became fragmented. Although there may not be enough data, these results support the notion that Sydney IVF Cleavage Medium K-SICM-100 is a suitable culture medium for the development of pre-implantation embryos to blatocyst stage. Further studies should be made to see if blastocyts or late morula stage- embryos would hatch and develop a trophoblast when cultured *in vitro* using this medium. The fact that this medium contains human serum albumin showed another quality that makes the guinea pig embryo a suitable model for studies on the human embryo.

The embryos collected from guinea pig 6 and 7 were not incubated since the experiment took place in Malta where only mice and rats are allowed to be used as laboratory animals (under E.U. regulations) and access to an incubator was not granted for the incubation of guinea pig embryos.

5.4 Guinea Pig VS Mouse

In comparison to mice, guinea pigs were easier to handle since they are bigger and good-natured. Application of the hormones intra-peritoneally was also easier and safer for the guinea pig compared to mice. The biggest advantage of using mice is that length of the cycle is 5 days and ovulation can easily be induced with the application of eCG (5-10 IU) and hCG (5-10 IU). Since guinea pigs have a complete cycle (luteal and follicular phase), ovulation cannot be induced but due to the unique vaginal closure membrane, oestrus can be easily detected and so superovulation can be induced. Another advantage of using mice is that the

procedure takes far less time and a good supply of oocytes or embryos can be made available on demand.

However, while collecting the embryos under the microscope it was evident that working with a bigger uterus was more comfortable and bigger embryos were easier to find; making the guinea pig ideal for studies concerning manipulation of the embryos, embryo transfer and *in vitro* fertilisation (IVF).

For superovulation, eCG and hCG were used on mice which are cheaper and more available for routine use on experimental animals. Unfortunately it is not known why these gonadotropins do not work on guinea pigs but it is probably due to the many different features of its reproductive system. Therefore, hMG was used for inducing superovulation in the guinea pig because of its similarity of guinea pig FSH receptor to human's (Suzuki *et al.* 2003) and also the hMG is produced by the pituitary, not the placenta. It seems like the guinea pig FSH receptor has a lower responsiveness to hormones with C-terminal repeats (both eCG and hCG have a long peptide in the C-terminus, so called C-terminal repeats). This structure may influence the affinity and/or efficacy of eCG and hCG to guinea pig FSH and LH receptors, respectively. These were some of ideas discussed with Dr.Suzuki via personal communication but none of them are confirmed yet.

The guinea pig is thought to be evolutionary distant from mice and rats, e.g. the alpha-subunits of gonadotropins are not so similar to mice and rats (Suzuki *et al.* 2002). The gonadotropin releasing hormone is very unique compared to those of other mammals; two amino acid residues are different in guinea pigs, (even though most of the other mammals have common sequences) and pregnant guinea pigs have progesterone binding proteins in the blood, while no other rodent has it (O.Suzuki via personal communication). These are the perfect examples that show how the molecular mechanisms in guinea pigs tend to be different from other rodents.

As discussed before, the guinea pig has many anatomical differences from other rodents too; such as the vaginal closure membrane, the absence of a tail, different placental structures, different embryo-implantation method, etc. In additions, the guinea pig has a nutritional difference which is the requirement of vitamin C as part of its diet, also not present in other rodents.

A lot of these features also happened to be similar in humans; these include long gestation period, small litter, non-seasonal polyoestrous, spontaneous ovulation, active corpora lutea, complete follicular and luteal phases and fertile post-partum oestrous. The placenta of the guinea pig also resembles that of humans, being a haemochorial placenta. Mice will abort their young once the ovaries have been removed, while guinea pigs manage to carry the foetus to term making them valuable to the study of endocrine control of human pregnancy (Terrill and Clemons, 1998). Both guinea pig and human blastocysts undergo the same interstitial-type implantation and produce syncytiotrophoblasts (Suzuki *et al*. 2003). These features, amongst others, make the guinea pig a better model for the study of human reproduction than mice.

6 Abstract

This experiment was designed to test out one of the methods proven to be successful in inducing superovulation and adjusting it to make it suitable for the use of common guinea pigs since laboratory specific pathogen-free guinea pigs were unavailable. The protocol chosen belongs to Suzuki *et al.* (2000) which is the least expensive and least time-consuming of the protocols when compared to Shi *et al.* (2000 a, b)'s protocol using inhibin vaccine, and Kosaka and Takhashi (1989)'s protocol using PMSG after implantation of progesterone tubing. A total of 7 guinea pigs were bought from different breeders and housed in a controlled environment while their oestrous cycle was observed. Vaginal smears were prepared to confirm the date of the ovulation, together with physical and behavioural changes. 5IU hMG were administered intraperitoneally to each guinea pig on day 12, 13 and 14, post ovulation. The female guinea pigs were introduced to a mature male while the vagina was open to allow copulation. An ovario- hysterectomy was performed to recover the uterus and ovaries and the embryos were flushed on day 4 of pregnancy. An average of 5.2 embryos was collected from 5 of the 7 guinea pigs used which means that the experiment was successful, as only 3.6 ± 0.1 embryos were flushed from guinea pigs which were allowed to ovulate spontaneously in Suzuki *et al.*'s experiment (1993) . Despite the fact that the CL were not counted, the mean number of embryos flushed during this experiment was very close to that of Suzuki *et al.* (2003) and Dorsch *et al.* (2009) collected on day 4 and day 4.5 post coitum respectively. The embryos flushed from guinea pig 1, 2 and 3 were incubated in a incubator set at 37.5˚C and 6.5% carbon dioxide gas for equilibration. Their development was monitored everyday for 5 days and 50-75% of the 8-16 stage morulae developed into blastocysts in the Sydney IVF Cleavage Medium K-SICM-100. These results confirmed the potential of the guinea pig as the ideal experimental model for human reproductive biology.

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8 Acknowledgements

I would like to start by thanking my tutor, Dr. Cseh Sandor, who found the time during his busy schedule to help me with the thesis topic of my choice and to provide me with all the necessary materials to achieve good results. His studies and lectures have inspired me to explore the wonderful discoveries in the department of reproduction.

Secondly, I would like to thank Dr. Osama Suzuki who always took the time to answer any questions I had regarding his work. I am grateful for all the quick and detailed replies; his insight was essential for my thesis.

I wish to tank Dr. Sara Kecskemeti who was extremely helpful with the detection of oestrus. I am also forever grateful to her for helping me with the ovario-hysterectomies rather than euthanizing the guinea pigs.

I would also like to thank Ms. Zsuzsanna Keresztes and Mr. Bence Somoskoi for their invaluable help with the laboratory work and for their costant patience throughout the experiment.

A big thank you goes to my family for all their support throughout this process; especially my mother who pushed me to further my studies by believing in me and assisting me financially, and my father who is constantly looking over me from above.

Thank you to all my friends who encouraged me when it got hard and believed that I will make it to the end; a special thanks goes to Steven Mercieca, Elaine Licari, Christina Formosa and Julia Zammit for taking care of the guinea pigs when I was unable to and Emma Vassallo Bianchi for proof-reading my thesis.

Last but not least, I would like to express my gratitude to my fiancé, Damian Falzon: I truly appreciate all the hours you spent listening to my ideas and issues, and the constant love and patience throughout these years. I could have never done it without you and I promise to use everything I have gained during this experience to better our future lives together.