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Implementation of the Intrauterine Artificial Insemination Technique in Lewis Rats for Harvesting Embryos used in Dental Tissue Engineering Projects

Aplicación de la técnica de inseminación artificial intrauterina en ratas Lewis para obtención de embriones utilizables en proyectos de ingeniería de tejidos dentales

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ABSTRACT
Background: The use of Lewis inbred rats in embryonic tissue transplantation experiments can present a challenge because of the fertility problems associated with the strain that require large production colonies to harvest sufficient tissue for the experiments, but this practice goes against the principle of reduction. *Purpose*: In order to maximize the fertilization of apt females an intrauterine artificial insemination technique (IUAI) was standardized in a Sprague Dawley outbred strain and later applied to the Lewis inbred colony. *Methods*: 41 Lewis rats to which estral stage determination was performed by impedance reading of the vaginal wall were inseminated. A midventral laparotomy was performed, the ovarian third of both uterine horns were located and gently elevated, and 350 µl of spermatozoid/0.9% saline solution were injected in each horn. *Results*: Even though the IUAI proved to be effective for use in outbred Sprague Dawley rats under these experimental conditions (average 5.08 embryos) at the time of sacrifice only 12 Lewis females (29.3%) were carrying embryos and the average embryos collected per female were 2.3. *Conclusion*: When the intrauterine artificial insemination technique was applied to the Lewis strain the number of pregnant females or the average embryo yield did not increase when compared to natural mating and therefore we do not recommend its use for this purpose.

KEY WORDS
Artificial insemination, Lewis inbred rats, Sprague Dawley, embryos, spermatozoids, embryonic stage, impedance.

THEMATIC FIELDS
Bioengineering, artificial insemination, laboratory animal sciences.

RESUMEN
Antecedentes: El uso de ratas endocriadas Lewis en experimentos de trasplantes puede representar un reto, por los problemas de fertilidad asociados con esta cepa, que implican mantener colonias de producción grandes, a fin de poder recolectar suficiente tejido para usar en los experimentos; sin embargo, esta práctica va contra el principio de reducción. *Propósito*: Estandarizar el protocolo de inseminación artificial intrauterina (IAIU) en una cepa exocriada Sprague Dawley y posteriormente aplicarla en la colonia de ratas Lewis, para maximizar el número de hembras aptas fertilizadas. *Métodos*: Se inseminaron 41 ratas Lewis a las que se les había determinado el estro por medio de impedancia vaginal. Se realizó una laparotomía medioventral para localizar y exponer el tercio ovárico de los cuernos uterinos, los cuales fueron cuidadosamente elevados y 350 µl de una solución de espermatozoides/suero fisiológico al 0,9% se inyectó en cada uno de los cuernos. *Resultados*: Bajo las condiciones experimentales presentes, la técnica de IAIU resultó efectiva en la cepa exocriada (promedio de 5,08 embriones). Al momento del sacrificio solo 12 hembras Lewis (29,3%) eran portadoras de embriones con un promedio de 2,3 embriones por hembra. *Conclusión*: Cuando se aplicó la técnica de inseminación artificial intrauterina a la cepa de ratas Lewis, el número de hembras preñadas o el promedio de embriones no aumentó en comparación con los resultados obtenidos por medio de cruces naturales. Por consiguiente, no recomendamos la aplicación de esta técnica para el propósito planteado.

PALABRAS CLAVE
Inseminación artificial, ratas endocriadas Lewis, Sprague Dawley, embriones, espermatozoides, etapa embrionaria, impedancia.

ÁREAS TEMÁTICAS
Bioingeniería, inseminación artificial, ciencias de los animales de laboratorio.

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INTRODUCTION

Experiments using animals as biological reagents have contributed greatly to science development and consequently to human wellbeing by increasing knowledge in most areas of biology, including developmental models, tissue engineering techniques, and therapy evaluation. In 1950, an inbred strain (Lewis) was developed from a Wistar nucleus colony by long term consecutive mating. The colony hosted in the animal housing facility at the Pontificia Universidad Javeriana Dental School has more than 60 years of inbreeding history and is being used as a source of inductive tissues in tooth bioengineering projects that involve harvesting embryos in the precise developmental stage when dental initiating signals are present.

Maintaining a rodent colony for experimental purposes under Specific Pathogen Free (SPF) conditions is not a minor enterprise but the quality of the obtained results compensates for the higher maintenance reducing result variability and animal distress related to optimal housing conditions while maintaining a small number of experimental subjects. The purpose of this study was aimed towards reducing the size of the colony while maintaining rigorous housing conditions in accordance with the guiding principles of laboratory animal use and welfare. On the other hand, the use of conventional animal colonies generates poor results and potential invalidation of the conclusions obtained. Implementing and maintaining an animal research program in accordance with the international guidelines for animal research has been the central commitment of the tissue engineering research team at our facility.

The inbred rat strain (Harlan Laboratories, Inc., Indianapolis, IN) is often used in transplantation experiments, because its high degree of homozygosity (>98%) (1,2) reduces the possibility of the transplant recipient having an immune reaction to the donor's organ. A major challenge for researchers using this strain relates to the decreased fertility when compared with other strains. The supplier reports that the mating success of this strain is one confirmed pregnancy in fifteen arranged mass pairings (6.7%). Reduced fertility likely results from several factors, including compromised genetic integrity of oocytes, poor sperm quality, low spermatogenic cell density, and low testosterone level of male rats (3). Because of its low fertility, generating sufficient offspring for experimentation can be a challenge.

Several alternatives intended to solve this problem have been considered. One of them implies maintaining a large breeding colony, but this notion goes against the principle of reduction under which researchers using animals are encouraged to design their experimental protocols to minimize the number of animals used (3,4). Another solution that reduces the number of experimental individuals involves increasing the likelihood that mating will occur when a reproductively active female is paired with a proven male by precisely determining the estral cycle stage by measuring vaginal wall impedance. In this case, when paired with a proven male, roughly 50% of the apt females are served (5). A third alternative to natural pairing that might increase the number of fertilized females is artificial intrauterine insemination. By applying this technique we could assure that all receptive females would be inseminated and therefore increase the number of cultured embryos maintaining a smaller colony.

Artificial insemination that has been successfully applied in outbred strains to obtain oocytes in fertility research (6) is accomplished by depositing a fresh or frozen-thawed suspension of spermatozoids manually inside the uterus thus overcoming the limitations associated with natural pairings and increasing pregnancies. Situations in which this procedure may prove helpful include low concentration of motile spermatozoids that are insufficient to achieve fertilization by natural pairing, or when the use of strains with low fertility rates is necessary for the accomplishment of specific experimental protocols (7). There are few reports indicating the use of artificial insemination in rats and none known that have been applied to the Lewis strain. Although this technique has gained importance, it has not been extensively used since it requires a great deal of surgical proficiency (8).

No matter which approach is used to obtain fertilization, the selection of apt females involves the determination of the estrous cycle phase, as female rats typically allow mating only during a certain phase of the estrous cycle (9). The estrous cycle of female Lewis rats lasts 4-5 d, as in other strains (10), and in a 5 day cycle consists of three distinct phases: proestrous (~1 d), estrous (~1 d) and diestrous (~2-3 d). Females are receptive to mating during the afternoon of the proestrous and morning of the estrous phase (2,11). To increase the likelihood that mating and conception will result from a pairing, the pairing should occur the night of the proestrous phase.

According to literature reports, standardization of estrous cycle stage determination indicating adequate timing for service can be performed in two ways; microscopic evaluation of vaginal smears or impedance readings. Recent reports indicate that vaginal wall impedance readings are a reliable alternative in the determination of estrous cycle stage (3,5,9), since many species including the rat present variations in the inherent electrical resistance of the vaginal wall during the estral cycle (9,10,12). This measurement is technically simpler to perform, quicker, and less subject to operator interpretation, exceeding 4 kΩ (kiloohms) at proestrous and dropping back during latter stages.

In the present study an alternative method to natural mating was used in order to assure fertilization of apt females. For this purpose an artificial insemination technique previously described in the literature (6) was standardized in outbred Sprague Dawley rats and later applied to the Lewis colony.

MATERIALS AND METHODS

Animals

This project was approved by the Universidad Javeriana Dental Research Center Ethics Committee as part of a separate study. All breeding pairs and embryos were used for other research purposes. All procedures used in this study complied with guidelines set forth in the International Guiding Principles for Biomedical Research Involving Animals (13). Laboratory procedures followed biosafety requirements for the management of biological specimens of Universidad Javeriana Dental Research Center (Universidad Javeriana Dental School Animal Facility).

Ten 8-week-old sibling pairs of male (150-225 g) and female (100-200 g) Lewis (LEW/SsNHsd) rats were purchased from Harlan Laboratories and mated monogamously to generate the foundation colony in our animal facility. The pairs were retired from the foundation colony after the third pregnancy and replaced with a new sibling pair. The offspring produced were used for breeding either in the foundation colony or in the production colony used for experiments.

Male and female rats in the production colony were group-housed (four or five rats per cage) in disinfected polycarbonate cages (36 cm × 49 cm × 21.2 cm; One Cage, Lab Products, Inc., Seaford, DE) according to age and sex. For breeding, rats were housed in pairs in polycarbonate cages (36 cm × 23.5 cm × 21.5 cm; One Cage, Lab Products, Inc.). All cages were covered with filter tops and contained sterile pine wood chips (Las Palmas Wood Deposit, Bogotá, Colombia). The cages were kept on a ventilated rack (One Cage, Lab Products, Inc.) with 50 total air changes per h of HEPA-filtered air. The room was maintained at a temperature of 22.0 ± 1.0 °C and a relative humidity of 50–65% on a 12-h:12-h light:dark cycle with lights on at 6:30 a.m. All animals were transferred to clean, disinfected cages with fresh bedding twice a week. Autoclaved rodent chow (Agrinal Rodentina, Buga, Colombia) and sterilized water were provided ad libitum. The water was supplemented with a vitamin–amino acid complex (Promocalier L, Calier Laboratories, S.A., Barcelona, Spain) once a week to compensate for nutrient loss upon chow sterilization.

Health monitoring was done twice a year. Rats were serologically negative for common bacterial and viral pathogens. Nasopharyngeal and cecal cultures analyzed by an independent laboratory were negative for dermatophytes and respiratory and enteric bacterial pathogens. Animals were also free of internal and external parasites. The members of the foundation colony were genotyped by an independent laboratory assuring the (LEW/SsNHsd) strain integrity.

A total of 12 female and 12 proven male Sprague Dawley (SD) rats were used for the standardization of the intrauterine artificial insemination technique (IUAI) technique and the same protocol was replicated in the 41 females and 60 proven male Lewis rats.

Estrous Cycle Phase Determination

Based on the results of a previous study (5), estrous cycle phase was determined by impedance readings. With the advice of the Reproductive Laboratory in the Pontificia Universidad Católica de Chile a group of approximately 31 SD female rats were sampled for 4 consecutive days and those females considered to be in proestrous were used the same day of the determination for the insemination procedure. Each night 3 females for a total of 12 were inseminated for the standardization of the protocol.

When the IUAI was going to be applied in the Lewis rat colony once every 2 weeks during a six month period, vaginal wall impedance was measured for 25 females in the production colony using an estrous cycle monitor (EC-40, Fine Science Tools Inc., North Vancouver, Canada) equipped with a 4.8-mm-diameter probe designed for use in rats. If the impedance was lower than

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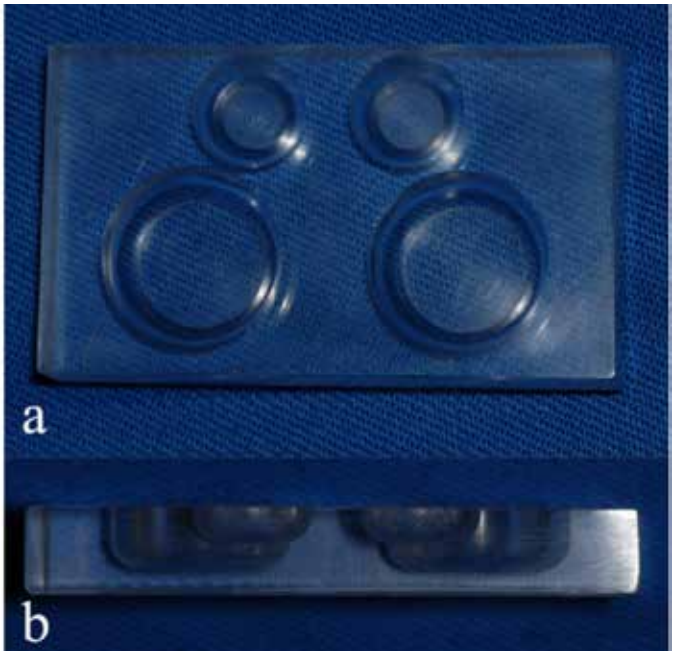
3.5 kΩ, it was determined that the rat was not in the proestrous phase and returned it to the colony, but if the impedance was 3.5 kΩ or greater, the rat was in the proestrous phase and could be inseminated. No more than 3 rats were inseminated per event. In total 41 acceptable impedance readings from approximately 75 rats over 26 weeks were obtained.

Spermatozoid Collection

Proven males were sacrificed at 21:00 h by asphyxiation with a mixture of CO_2 and air followed by cervical dislocation. The abdomen was shaved and disinfected with a 0.2% clorhexidine solution. A midventral incision was used to expose the abdominal cavity and a radical orchiectomy including the epididymis and deferent ducts. The epididymal tail and the vas deferens were separated and placed in a petri dish containing 0.9% saline solution at 37 ºC. The tail of the epididymis was used, due to the fact that the epididymal transit favors intrinsic biochemical and physiological modifications that result in the acquisition of progressive motility and the ability to undergo capacitation required for fertilization protection, maturation, concentration and it is there that spermatozoids are stored until ejaculation (14). Under stereomicroscopic magnification, all remaining adipose tissue and blood vessels were carefully dissected and eliminated to avoid lipid contamination and the spermicide action of blood. A sterile collection tray with two diffusion and two discarding wells was fabricated in transparent acrylic resin for storage of the organ sections and the spermatozoid solution (Figure 1).

Figure 1
Sterile Collection Tray

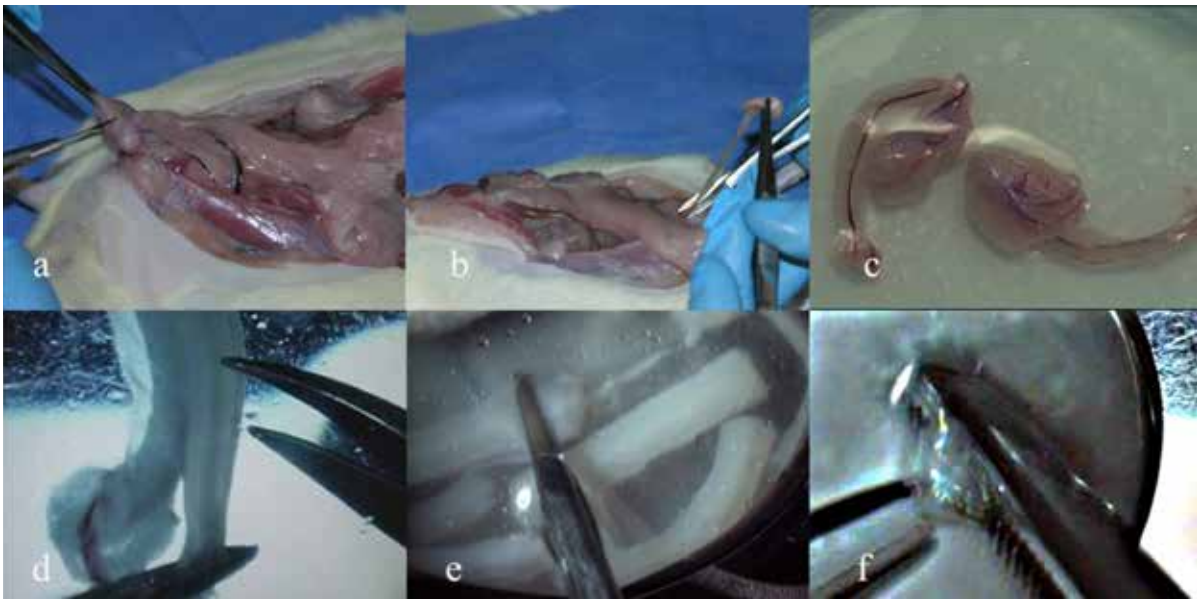


a) A sterile collection tray with two large diffusion (1.3 ml) and two small discarding wells (0.5 ml) fabricated in transparent acrylic resin facilitates the sperm collection procedures when used under a stereomicroscope with diascopic illumination. b) Frontal view of the collection tray.

The clean vas deferens were cut in 0.5 cm sections and together with the distal portions of the epididymal tails were placed in the diffusion wells

containing 1 ml of 0.9% saline. One epididymal tail and vas deferens from each of the proven males was placed into each one of the two diffusion wells to balance the sperm concentration in the solution used for the insemination. Spermatozoids were extracted from the vas deferens sections by pinching one end with splinter forceps and using another splinter forceps, from which the serrations had been completely eliminated leaving a smooth surface, to squeeze the duct fragment and extract the spermatozoids. The distal tail portion of the epididymis was partially cut with microdissection scissors and macerated with tissue forceps (Figure 2). The collection tray was placed inside an airtight container to avoid water evaporation and stored at 37 ºC and 5% CO_2 prior to use, allowing for diffusion of the spermatozoids. Preceding intrauterine injection, the tissue fragments were eliminated from the collection tray's diffusion well and transferred to the discarding wells. The percentage of motile spermatozoa was assessed visually by direct observation under a light microscope at 10 X magnification. Over 80% motility was preserved at 1 h after collection and sperm concentration fluctuated between (40-50x106) spermatozoids/ml.

Figure 2
Preparation of the Spermatozoid/0.9% Solution

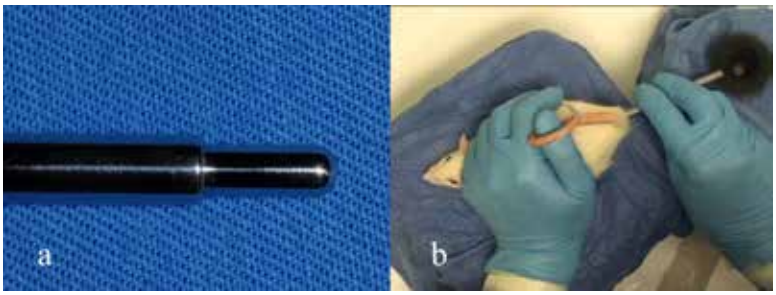


a), b), and c) The epididymal tail and the vas deferens were separated and placed in a petri dish containing 0,9% saline solution at 37 ºC. d) All remaining adipose tissue and blood vessels were carefully dissected and eliminated under stereomicroscope to avoid lipid contamination and spermicide action of blood. e) Spermatozoids were extracted from the ducts by pinching one end with splinter forceps and using another splinter forceps to which the serrations had been completely eliminated achieving a smooth surface to milk the spermatozoids out of the duct. f) The epididymal distal tail portion was partially cut with microdissection scissors and squeezed with tissue forceps.

Pseudopregnancy by Mechanical Vaginal Stimulation

In order to achieve pseudopregnancy, proestral females were stimulated twice in 2 min periods with a ten minute interval between them by introducing a 4.8 mm diameter highly polished stainless steel rod lubricated with sterile mineral oil inside the vagina and placing the rod against a vortex agitator (Figure 3). The female's response to the mechanical stimulation was recorded as positive, moderate or absent. A rise in circulating estrogens during the proestrous morning activates the neural circuits for lordosis by acting on brain nuclei (2). If the rat presented the lordosis reflex, the response was considered positive. Only females with positive or moderate responses were inseminated.

Figure 3
Pseudopregnacy Induction



a) 4.8 mm diameter highly polished metal rod. b) Proestral females were stimulated twice for 2 min within a ten minute interval by introducing the metal rod lubricated with sterile mineral oil inside the vagina and placing it against vortex agitator.

Midventral Laparotomy for Intrauterine Insemination

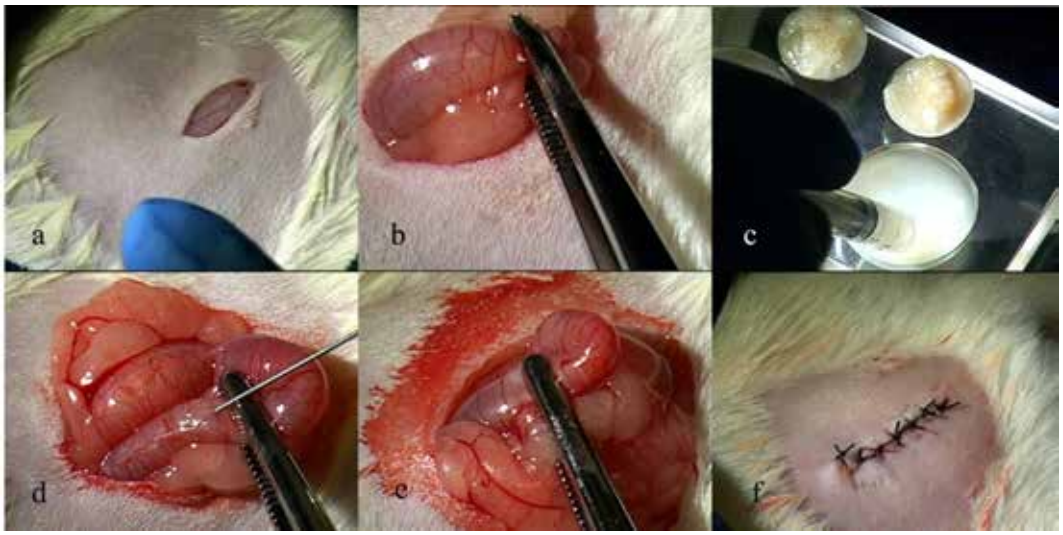
After pseudopregnancy was induced by mechanical stimulation, the proestral female was placed in an induction chamber and anesthetized (Ohmeda vaporizer model Fluotec 4) using 3% Isoflurane (FORENE®) with an oxygen flow between 1.5 and 2 l/min. After induction, the abdomen was shaved and disinfected with 0.2% clorhexidine solution. The animal was reintroduced in the induction chamber and afterwards connected by means of a rodent mask to an open anesthesia circuit and maintained with 1.5-2% Isoflurane and a 1.5-2 l/min oxygen flow. Heart rate and oxygen saturation were monitored (Ohmeda Biox 3700 pulsoximeter) with the pulsoximeter's probe (Ohmeda Veterinary Pulse Oximeter Lingual Sensor) attached to the animal's tail. At normal operating room ambient temperature (21 ºC), small rodents can rapidly lose body temperature due to heat dissipation, therefore a thermic blanket was placed on top of the surgical table to avoid hypothermia during the surgical procedure.

Placing the animal in a decubitus supine position, a 1.5 cm midventral incision was performed over the Linea Alba 3 cm caudal of the xiphoid appendix. A layer dissection was performed until exposure of the parietal peritoneum, which was then cut in order to gain access to the peritoneal cavity. Once inside the cavity, the ovarian third of one uterine horn was located and gently elevated with tissue forceps. Using an insulin syringe with a 25 gauge needle, 350 µl of spermatozoid/0.9% saline solution were injected, the needle was slowly withdrawn, and slight pressure was applied over the perforation for one minute (Figure 4). The inseminated uterus was reintroduced into the peritoneal cavity, and the procedure was repeated in the contralateral uterine horn. After completing the insemination, the muscular and fascial layers were closed in one plain with a simple continuous suture and the skin with simple interrupted sutures both using 4-0 silk. Postsurgical analgesia was achieved by oral administration of a morphine/water solution (2.5 mg/kg) dosed every 6 h for 24 h.

Female Sacrifice and Embryo Count

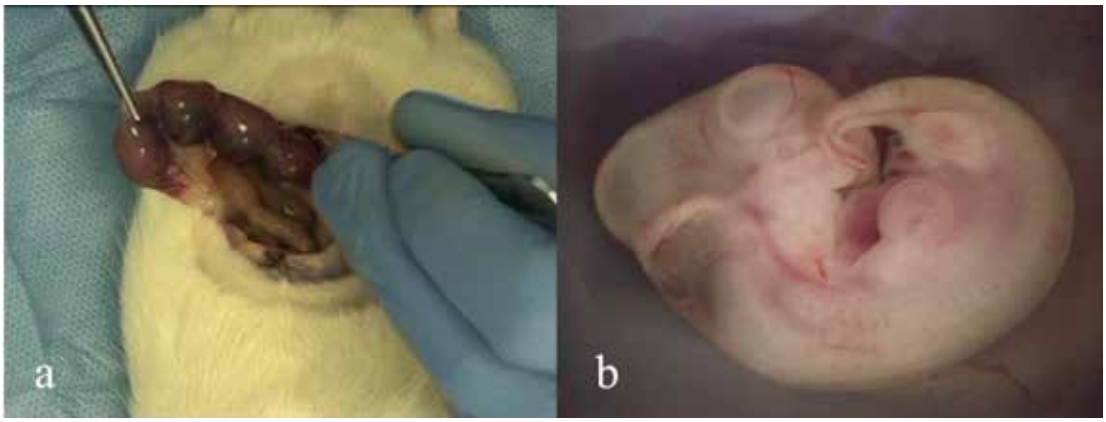
When it was time to collect the embryos at embryonic day 12.5-13.5, inseminated females were euthanized by asphyxiation with a mix of carbon dioxide and air followed by cervical dislocation. A laparotomy was used to expose the uterine horns which were dissected and placed in phosphate-buffered saline solution. The number of viable embryos collected from each female were counted and recorded. The embryos were used as a source of inductive tissues in a tooth bioengineering study (Figure 5).

Figure 4
Injection of the Spermatozoid Solution



a) Midventral incision to gain access to the abdominal cavity. b) Elevation of the ovarian third of one uterine horn. c) Loading the insulin syringe from the diffusion well containing the spermatozoid solution. d) Injection of the 350 µl of spermatozoid solution in the distal portion of the uterine horn. e) Slight pressure applied over the perforation. f) Final closure of the surgical wound.

Figure 5
Embryo Harvest



a) Embryo harvesting by means of a laparotomy. b) Lewis E13.5 embryo obtained by IUAI used as a source of inductive tissues in a tooth bioengineering study.

Statistical Analysis

All data collected were analyzed using the Stata 10 program (Stata Corp., College Station, TX). Correlations between receptivity and embryo average when the IUAI was applied to the inbred strain were evaluated with the Chi-square test ($p<0.05$). The Wilcoxon-Mann-Whitney test was used to compare the application of the IUAI techniques between strains ($p<0.05$).

RESULTS

Standardization of the IUAI was performed in 12 female SD rats. All individuals showed positive response to mechanical stimulation for induction of pseudopregnancy, and 9 presented viable embryos upon sacrifice. The average of viable embryos per pregnant female was 5.08 (SD±4.5) (Table 1).

Table 1
Summary of the application of the IUAI technique to the outbred Sprague Dawley (SD) females

Total females SD	12
Number of SD females pregnant with viable embryos	9 (75%)
Average of collected embryos from SD rats	5.08 (SD±4.5)

The same protocol was used in 41 Lewis rats to which estral stage determination was performed by impedance reading of the vaginal wall. Of the selected females, 85.4% showed positive response to mechanical stimulation to achieve pseudopregnancy. At the time of sacrifice 12 females (29.3%) were carrying embryos and the average embryos collected per female were 2.3 (SD±1.8). Table 2 shows the total female distribution according to the impedance readings in kiloohmio.

Table 2
Collected Lewis (L) Embryos using the Intrauterine Artificial Insemination (IUAI) Technique. Impedance values are registered in kΩ

	Impedance (kΩ)			Total
	3.5 a 3.9	4 a 5	> 5	
Total females L	5 (12.2%)	21 (51.2%)	15 (36.6%)	41 (100%)
Number of L females pregnant with viable embryos	2 (40.0%)	5 (23.8%)	5 (33.3%)	12 (29.3%)
Average of collected embryos from L females	1.5 (sd 0.7)	3 (sd 2.3)	1.8 (sd 1.3)	2.3 (sd 1.8)
Total number of embryos collected from pregnant L females	3 (24.6%)	15 (67.2%)	9 (8.2%)	27

The Chi square (χ^2) test showed no positive correlation between embryo number and receptivity at the moment of pseudo pregnancy (Table 3).

Table 3
Correlations between Receptivity and Embryo Average in Embryo Carrying Inbred Lewis (L) Strain

Receptivity	n	%	p-value	Average
Positive	10	28.6	0.813	2.4 (sd 1.9)
Mild	2	33.3		1.5 (sd 0.7)

The results of the Wilcoxon-Mann-Whitney test showed a statistically significant difference (p=0.0070) when comparing the results of the IUAI technique applied to the SD and Lewis rats, evidenced by a smaller amount of recovered embryos in the Lewis strain.

DISCUSSION

Although *in vitro* and *in vivo* fertilization are powerful tools for restoring conserved sperm from stocked males in rats, they are not used extensively for efficient production of rat offspring because the techniques require a great deal of surgical skill (8).

Results obtained with the IUAI in the outbred SD strain suggest that the protocol is reliable and can be used to assure fertilization of apt females (75% of the inseminated SD females were pregnant at the time of sacrifice) but it has

inherent limitations. One drawback refers to the fact that when naturally mated, the average litter size of the reference strain used for protocol standardization is 11 as reported by Harlan Laboratories, but when inseminated, the average drops to 5.08. This shows that even though the rate of pregnancies is adequate (75%), the litter size when inseminated with this protocol tends to be smaller when compared to natural mating (7.2). This same tendency is seen when the IUAI is applied in the Lewis strain. The more restrictive condition relates to the fact that proven males have to be sacrificed, and if otherwise used for natural mating, they could be preserved throughout their active reproductive life. Artificial Insemination would only be justified if the cryopreservation of a specific and valuable individual's sperm is desired once it reaches the limit of its reproductive life.

When the IUAI was applied to inbred Lewis rats we found a marked decrease in the average of collected embryos (2.3) when compared to natural mating of the same strain (7.2) as reported in a previous study (5). In this same study, the authors report that of 90 females with proven service, 55 were embryo carriers while 41 of the inseminated L females (equivalent to the proven service females of the referred study) only 12 (29.2%) were pregnant at the time of sacrifice showing that in this strain and under these experimental conditions, artificial insemination is less effective than natural mounting. Embryo yield was also greatly decreased and amounted to 7.79% of the reported yield obtained with natural mating.

When elevating the uterine horns during the surgical procedure, the presence of luminal fluid, characteristic of the proestral stage, was detected. The control of the fluid environment of the uterus is essential for a number of key reproductive events, including sperm and embryo transport, development and implantation (15). The cervical canals of the rat are closed by a sphincter-like action at estrus and the horns become distended as a result of estrogen stimulation. They become empty after oophorectomy and become distended with a fluid similar to that found at estrus following estrogen administration (16). Estradiol induces a fluid secretion containing sodium and potassium, into the lumen of the uterus affecting the volume of intraluminal fluid in the rat (15). Reabsorption of fluid from the uterine lumen under the influence of progesterone presumably facilitates the reduction in volume of the uterine cavity, preceding the attachment reaction (17).

In some individuals, liquid backflow through the vagina after the injection of the spermatozoid occurred when inseminating both the SD and Lewis rats. This backflow might be related to the timing of the estrogen surge prior to insemination and might reduce the volume of available spermatozoid solution as well as the uterine fluid, therefore decreasing the efficiency of the insemination procedure. If the insemination is not performed at the appropriate time, the cervical canal may be open and thus liquid backflow may occur.

In vivo, spermatozoa require the physiological changes known as capacitation in the female reproductive tract after ejaculation (18). Although the detailed molecular mechanisms of capacitation remain unknown, it has been shown in several species that protein tyrosine phosphorylation in sperm involves a signal transduction cascade (18). The use of saline solution as the solvent for the spermatozoids might affect uterine fluid composition reducing its capacitation efficiency. Another important consideration relates to the fact that the ejaculated spermatozoa aspirated from the rat uterus have a distinct gel coating of seminal plasma constituents and the gelation of the seminal plasma constituents is induced by bicarbonate present in the uterine fluid. This coating probably serves to inhibit the decapitating activity of the uterine fluid peptidase (19) which adheres to the spermatozoa and may favor ovum penetration or assist in capacitation of spermatozoa in the uterus. Since the spermatozoids solution injected lacks these constituents, the fecundation process might be negatively affected.

It is important to emphasize that the complex fecundation processes are directly controlled by hormone concentration in the female's reproductive tract influencing spermatozoid capacitation that occurs at a specific moment depending on the species. The exact timing of hormonal concentrations and spermatozoid capacitation may also be strain specific thus explaining the difference in the results obtained.

The collected spermatozoids were not supplemented with a base media since the purpose of such additions is to maintain sperm motility and membrane integrity against the adverse effects of freezing-thawing in cryopreservation protocols (20) that are implemented in the conservation of genetic sources of mutant or transgenic rats.

A prior study suggested the use of an intraperitoneal injection of oxytocin (1/800 IU) immediately prior to non-surgical artificial insemination of Wistar rats with frozen-thawed spermatozoids to increase the number of pups at birth, suggesting oxytocin induces uterine

contractions that might play an important role in spermatozoid transport. Nonetheless the authors mention that all individuals subjected to non-surgical insemination with fresh spermatozoids became pregnant indicating that the use of oxytocin is not necessary when high motility spermatozoids are used (21). For this reason we did not include an oxytocin injection protocol in this study.

As previously described in the literature pseudopregnancy may be produced by mechanical stimulation by means of a vasectomized male. For this purpose a proestral female and a vasectomized male are paired from 16:00 to 22:00 hours the same day of the insemination procedure (18,20). Other authors report the use of mechanical stimulation with a glass rod 1 hour prior to the procedure (21). Both approximations have proven to be adequate but the use mechanical stimulation with a rod allows for reduction of the number of males in the colony used for the sole purpose of achieving pseudopregnancy.

Finally in order to simplify the protocol for preparing the spermatozoid/saline solution a transparent acrylic collection tray was designed and constructed so it would fit inside an airtight 100% humidity container. The standard protocol solution uses mineral oil to create a bubble of saline solution inside of which the squeezing of the vas deferens and maceration of epididymal tails is done. The purpose of the bubble is to reduce evaporation of the solution during storage but contamination of the spermatozoid solution with mineral oil is frequently seen upon injection inside the uterus. The use of the collection tray expedites the sperm solution preparation.

CONCLUSIONS

The use of *in vitro* and *in vivo* fertilization for the restoration of conserved sperm is a powerful tool that has progressively gained acceptance. However, these techniques are not used extensively for efficient production of rat offspring, because they require a great deal of surgical skill (21) and also because the low embryos yield when compared to that obtained by natural mating. As previously reported (5) of 187 natural L pairings, 55 resulted in pregnancy (29.4%), and 397 viable embryos were collected, yielding an average of 7.2 viable embryos for each pregnant rat. Even though the IUAI proved to be effective for use in outbred SD rats (SD average 5.08 embryos) under these experimental conditions, when applied to an inbred L strain it did not increase the number of pregnant females or the average embryo yield when compared to natural mating (2.3 for L AIUI and 7.2 for Lewis natural matings) and therefore we do not recommend its use for this purpose.

RECOMMENDATIONS

Colony size reduction and increase of embryo yield when using the L rat strain for bioengineering projects can be achieved through methods other than the AIUI technique, such as superovulation or estrous cycle synchronization. The application of these methods to an inbred strain should be evaluated.

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