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Evaluation of sperm methylation as indicator of germ line alterations after successive applications of a cryopreservation program in *Oryctolagus cuniculus*

Master's Thesis by
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Abstract

The embryo early vitrification and its subsequent transfer is one of most common Assisted Reproductive Technologies (ART). This technology has allowed to improve the reproductive efficiency, crossbreeding and germplasm preservation programs. Multiple studies in livestock animals, even in humans, have demonstrated a set of alterations in both pre- and postnatal level, and the possible inheritance of these modifications in following generations.

Preimplantation embryo is a critical period, since is when most of epigenetic reprogramming take place, and the early embryo vitrification could be modifying this methylation pattern. Therefore, the aim of the current work was to evaluate the accumulative effects of two consecutive embryo vitrification procedures on rabbit (*Oryctolagus cuniculus*) embryo survival rate, rabbit sperm traits and epigenome profile, and analyse its possible inheritance. To this end, two populations were developed: vitrified embryos transferred to surrogated mother (VT) and naturally-conceived animals (NC) through two generations.

All the descendants who were born were healthy, supported by the haematological analysis, and ejaculates from VT group did not show reproductive effectiveness differences respect to NC group. Nevertheless, a 31% decrease of survival rate from first to second generation was registered, indicating a possible negative accumulative effect as consequence of successive cryopreservation. When the sperm epigenome profile was analysed, revealed a level of methylation higher in gene body than promoter regions. No difference were observed between NC and VT groups in the same generation, but a second round of vitrification (VT_G1 vs VT_G2) registered a higher level of methylation in all the regions of interested (ROI) analysed. A total of 1049 and 987 differentially methylated regions (DMRs) were obtained in G1 and G2, respectively. The functional analysis revealed enriched altered Go Terms in relation to protein metabolism, carbohydrate binding and oxidative phosphorylation. From those 1049, 387 remained altered in both generations, suggesting a possible intergenerational transmission of epigenetic marks as a consequence of embryo cryopreservation procedure.

In summary, even no differences in health or sperm quality were observed, an accumulative decrease of survival rate was reported after two rounds of cryopreservation. Additionally, a set of DMR were altered in both generations what could be indicating that the embryo cryopreservation is imprinting an epigenetic mark in the germinal cell, with consequence in the reproductive traits.

Keywords: sperm quality; rabbit; epigenetic; TRA; methylation; vitrification; cryopreservation

Resumen

La vitrificación de embriones y su posterior transferencia se utilizan ampliamente entre las tecnologías de reproducción asistida (ART) en la producción ganadera. Este procedimiento permite mejorar la eficiencia reproductiva, los cruzamientos y los programas de conservación del germoplasma. Lejos de considerarse neutral, debido a los múltiples estudios en animales de granja que han demostrado que la manipulación de embriones ha alegado muchos resultados adversos a nivel pre- y postnatal, se ha demostrado la posible herencia de los fenotipos adversos.

Embriones preimplantacionales se encuentran en un período crítico, ya que es cuando la mayor parte de la reprogramación epigenética tiene lugar. Además, la vitrificación embrionaria temprana podría promover muchos patrones alterados de metilación, considerados marcas epigenéticas. Por lo tanto, el objetivo del trabajo actual será evaluar los efectos acumulados de dos consecutivos procesos de vitrificación embrionaria sobre la tasa de supervivencia de embriones de conejo (*Oryctolagus cuniculus*), las características del esperma de conejo y su perfil epigenético, y su posible transmisión a lo largo de dos generaciones. Para este fin, se desarrollaron dos poblaciones: embriones vitrificados y transferidos a la madre de acogida (VT) y animales concebidos naturalmente (NC).

Al nacer, fue calculada la tasa de supervivencia del embrión y un descenso de la supervivencia en la segunda generación fue demostrado. Todos los descendientes que nacieron estaban sanos, respaldados por el análisis hematológico, y la eyaculación del grupo VT no mostró diferencias de efectividad reproductiva con respecto al grupo NC. Esta falta de diferencias podría deberse a la plasticidad fenotípica. El perfil epigenético del esperma en edad adulta fue evaluado y considerando la densidad de metilación en las regiones genéticas, el nivel de metilación en el cuerpo del gen es más elevada que en el promotor. Además, diferencias epigenéticas entre animales VT y NC en el metabolismo de las proteínas, la unión a carbohidratos y la fosforilación oxidativa se han registrado en ambas generaciones, lo que puede contribuir en las alteraciones reproductivas observadas.

Los hallazgos actuales sugieren que la criopreservación de embriones, como programa de preservación del germoplasma, puede promover consecuencias adversas sobre la tasa de supervivencia de embriones, correlacionadas con alteraciones moleculares a nivel epigenético en las células germinales parentales, que podrían afectar a las generaciones venideras.

Palabras clave: calidad espermática; conejo; epigenética; TRA; metilación; vitrificación; criopreservación

Resumeixen

La vitrificació d'embrions i la seua posterior transferència s'utilitzen àmpliament entre les tecnologies de reproducció assistida (ART) en la producció ramadera. Aquest procediment permet millorar l'eficiència reproductiva, els creuaments i els programes de conservació del germoplasma. Lluny de considerar-se neutral, a causa dels múltiples estudis en animals de granja que han demostrat que la manipulació d'embrions ha al·legat molts resultats adversos a nivell pre- i postnatal, s'ha demostrat la possible herència dels fenotips adversos.

Embrions preimplantacionals es troben en un període crític, ja que és quan la major part de la reprogramació epigenètica té lloc. A més, la vitrificació embrionària primerenca podria promoure molts patrons alterats de metilació, considerats marques epigenètiques. Per tant, l'objectiu del treball actual serà evaluar els efectes acumulats de dos consecutius processos de vitrificació embrionària sobre la taxa de supervivència d'embrions de conill (*Oryctolagus cuniculus*), les característiques de l'esperma de conill i el seu perfil epigenètic, i la seua possible transmissió al llarg de dues generacions. Per a aquest fi, es van desenvolupar dues poblacions: embrions vitrificats i transferits a la mare d'acolliment (VT) i animals concebuts naturalment (NC).

En nàixer, va ser calculada la taxa de supervivència de l'embrió i un descens de la supervivència en la segona generació va ser demostrat. Tots els descendents que van nàixer estaven sans, recolzats per l'anàlisi hematològica, i l'ejaculació del grup VT no va mostrar diferències d'efectivitat reproductiva respecte al grup NC. Aquesta falta de diferències podria deure's a la plasticitat fenotípica. El perfil epigenètic de l'esperma en edat adulta va ser evaluat i considerant la densitat de metilació a les regions genètiques, el nivell de metilació en el cos del gen és més elevat que en el promotor. A més, diferències epigenètiques entre animals VT i NC en el metabolisme de les proteïnes, la unió a carbohidrats i la fosforilació oxidativa s'han registrat en les dues generacions, la qual cosa pot contribuir en les alteracions reproductives observades.

Les troballes actuals suggereixen que la criopreservació d'embrions, com a programa de preservació del germoplasma, pot promoure conseqüències adverses sobre la taxa de supervivència d'embrions, correlacionades amb alteracions moleculars

a nivell epigenètic en les cèl·lules germinals parentals, que podrien afectar les generacions esdevenidores.

Paraules clau: Qualitat espermàtica; conill; epigenètica; TRA; metilació; vitrificació; criopreservació

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1. Introduction

The use of Assisted Reproductive Technologies (ART) in livestock production have leaded a relevant improve of the reproductive efficiency, genetic programmes and crossbreeding (Davis & White, 2020; Hansen & Siqueira, 2018). Close to 1.5 million transferrable embryos were produced by ART in 2017, being a 60% of them frozen/thawed embryos (Viana, 2018). The common ARTs in livestock production such as superovulation, artificial insemination (AI), embryo transfer (ET) or cryopreservation are considered low invasive (Thomopoulos et al., 2017), but it has been reported that embryo exposition to a different environment can determinate its viability or postnatal outcomes (Saenz-de-Juano et al., 2012; Garcia-Dominguez et al., 2018). In cattle (*Bos Taurus*), disorders have been assigned to superovulation and ET, such as longer gestations (Wagtendonk-de Leeuw et al., 2000) or foetal overgrowth associated with perinatal complications (Hansen & Siqueira, 2018; Ramos-Ibeas et al., 2019). Alterations in placenta vascularization due to *in vitro* embryo manipulation have been observed in sheep (*Ovis aries;* Duranthon & Chavatte-Palmer, 2018). *In vitro* porcine embryos have presented a minor quality, with a higher cell damage and apoptosis (Ramos-Ibeas et al., 2019). In rabbit (*Oryctolagus cuniculus*), the embryo manipulation has reported higher mortality rates at pre-natal stage and modified phenotype at adult stage (Vicente et al., 2013; Lavara et al., 2015).

In animal production, the gamete or preimplantation embryo cryopreservation is one of the most common ART, being applied to different purposes such as germplasm preservation programs (Marco-Jiménez et al., 2018), diffusion of the lines to different countries (García et al., 2000) and preserve genetic resources of interest associated with genetic selection programs (Lavara et al., 2011). Sperm and embryo cryopreservation not only allow to develop genetic programmes, but also the preservation of the fertility and viability over time (Goto et al., 2002; Wong et al., 2014) or make possible a genetic market (Hafez, 2015). Unlike *in* vitro fecundation or *in vitro* culture that try to imitate the physiological conditions, the germplasm cryopreservation involves the exposition to toxic cryoprotectant solutions and extreme-low non-physiological temperatures (Viudes-de-Castro et al., 2014; Wong et al., 2014). Embryo cryopreservation protocols include the use of several ART: superovulation, AI or *in vitro* fertilization, *in vitro*

handling, cryopreservation-thawing step and ET (Duranthon & Chavatte-Palmer, 2018). Therefore, in addition to the possible effects induced by the embryo cryopreservation step *per se*, we have to consider the cumulative effects of the other ART (Vicente et al., 2013). Then, to cover possible synergies between different ART stressors, in the current study we compared our cryopreserved group with naturally-conceived animals.

Preimplantation embryo is a critical period, since is when most of epigenetic reprogramming take place (Salilew-Wondim et al., 2018; Lou et al., 2019). In fact, previous studies have reported altered epigenetic kinetic in *in vitro* culture rabbit embryos at preimplantation stage (Canovas et al., 2017) or in adult human liver after chemical an physical variations promoted by ART procedures (Duncan et al., 2014). Alterations of the methylation pattern have been observed not only in the somatic cells, but also in spermatogenic cells or in the germinal cells (spermatozoa) in offspring from mice that have reported epigenetic reprogramming delays after female gonadotropin stimulation (Canovas et al., 2017; Stouder et al., 2009). Therefore, the epigenetic marks created after TRAs may be transferred to the following generations, affecting their offspring metabolic phenotype (Illum et al., 2018). In cattle, embryo *in* vitro culture induced epigenetic marks correlated to defects in focal adhesion and metabolic pathways (Salilew-Wondim et al., 2018). In rabbit, the early embryo cryopreservation revealed epigenetics marks in adult liver related to lipid metabolism, oxidative phosphorylation and dysregulation in zinc (Garcia-Dominguez et al., 2020b). Finally, Novakovic et al. (2019) evidenced that in humans the differential methylation is age-related and some of the modifications could be corrected in adulthood, but some of them could be transmitted to the next generations via the germ line (Lavara et al., 2014; Ramos-Ibeas et al., 2019; Rodríguez & Sánchez, 2019; Garcia-Dominguez et al., 2020c).

Few studies in animal production have been performed at inter-generational level. In order to evaluate the possible transmission of these marks to the next generations, in the current study the animal model used was the rabbit, which early embryo vitrification has reported adverse outcomes both pre- and postnatal stage (Lavara et al., 2014; Garcia-Dominguez et al., 2020a, 2020b, 2020c). At pre-natal stage, lower embryo implantation or foetal survival were observed (Mocé et al., 2010; Marco-Jimenez et al., 2013), meanwhile at long term, was registered a lower growth rate and

adult weight (Garcia-Dominguez et al., 2018). Furthermore, Saenz-de-Juano et al. (2014a; 2015) demonstrated that rabbit vitrification induced transcriptome and proteome changes of the foetal placenta, being associated to the higher gestational losses in the vitrified group. Therefore, the aim will be to evaluate the accumulative effects of consecutive embryo vitrification procedures on rabbit sperm traits and epigenome profile along two generations.

2. Materials and methods

All chemicals in this study were reagent-grade and purchased from Sigma–Aldrich Química S.A (Madrid, Spain), unless stated otherwise.

2.1. Animals

The experiment was carried out with rabbits from genetic maternal line based on New Zealand white from the ICTA Universitat Politècnica de València (UPV). All animals were handled according to Directive 2010/63/EU EEC for animal experiments and reviewed and approved by the Ethical Committee for Experimentation with Animals of the Univesitat Politècnica de València, Spain (research code: 2018/VSC/PEA/0116).

2.2. Experimental Design

Initially, 2 experimental populations were developed: one from vitrified ET to the surrogate mothers (VT) and other from naturally-conceived animals (NC) (Figure 1).

Females were AI with semen of unrelated males from the same strain. In VT group, 3 days after AI, embryos were recovered, vitrified and then transferred to surrogate mothers by laparoscopy. A total of 73 NC animals and 65 VT animals were conceived to create first generation (G1). At birth, survival rate was calculated and when they arrived at the reproductive age, sperm quality was evaluated and before crossing them blood samples were collected. A total of 162 and 91 animals from the NC and VT, respectively, were created in second generation (G2). Survival rate, sperm traits and blood samples were also collected in G2 at the reproductive age.

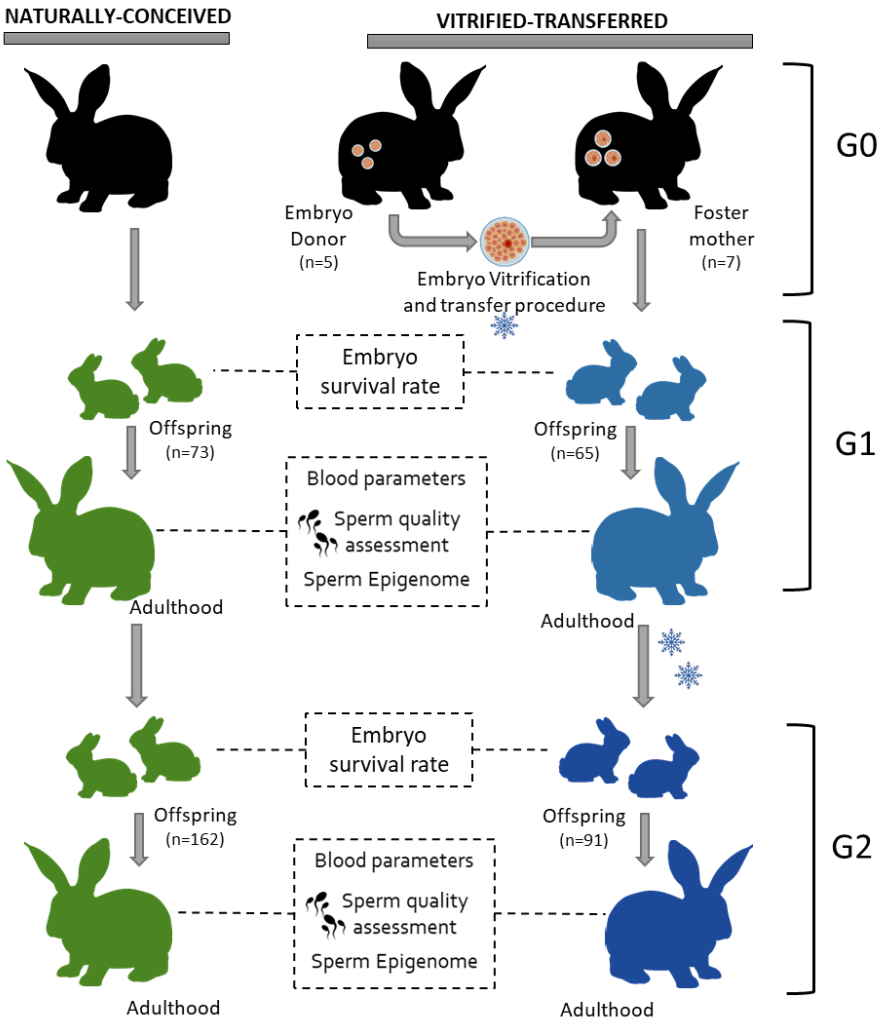

Figure 1. Experimental design. Vitrified-transferred population arises from vitrified embryos, meanwhile naturally-conceived population was generated without embryo manipulations. At birth, the embryo survival rate was calculated and at adulthood, the seminal traits were evaluated and sperm samples were collected to Genome-wide DNA methylation analysis (Epigenome).

2.2.1 *In vivo* embryo production and collection

5 donors from G0 were AI with a pooled sperm from 5 unrelated fertile males at a rate of 10×10^6 spermatozoa/mL in Tris-citric- glucose extender (Viudes-de-Castro & Vicente, 1997). Motility was examined at room temperature under a microscope with phase-contrast optics at 40x magnitude. Only those ejaculates with >70% motile sperm (minimum requirements commonly used in AI) were pooled (Marco-Jiménez et al., 2010). Immediately after insemination, super-ovulation was induced by an intramuscular injection of 0.75 µg/Kg of corifolitropin-α (Elonva, Merck Sharp & Dohme B.V.). Females were euthanized at 72 h post-insemination and embryos were collected

at room temperature by flushing of each oviduct and uterine horn (the first one third) with 5 ml of embryo recovery media, consisting of pre-warmed solution (≈20-25 °C) of Dulbecco's Phosphate-Buffered Saline (DPBS) solution supplemented with CaCl2 (0.132 g/L), 0.2% (v/v) of bovine serum albumin (BSA) and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL and amphotericin B 0.25 µg/mL). After recovery, morphologically normal embryos (morulae) with homogeneous cellular mass, mucin coat and intact zona pellucida were used for vitrification procedure. Same procedure was performed in the following generation (G1) but using as donor 16 females.

2.2.2. Vitrification and warming procedure

Embryos were vitrified and warmed using the methodology described by Vicente et al. (1999). Embryos were vitrified in two-step addition procedure at room temperature (≈20-25 °C). In the first step, embryos were placed for 2 min in an equilibrium solution consisting of 10% (v/v) dimethyl sulphoxide (DMSO) and 10% (v/v) ethylene glycol (EG) in DPBS supplemented with 0.2% of BSA. In the second step, embryos were suspended for 30 seconds in a solution of 20% DMSO and 20% EG in DPBS supplemented with 0.2% of BSA. Then, embryos suspended in vitrification medium were loaded into 0.125 mL plastic straws (French ministraw, IMV, L'Aigle, France) adding 2 sections of DPBS at either end of each straw, separated by air bubbles. Finally, straws were sealed and plunged directly into liquid nitrogen. Warming was done by horizontally placing the straw 10 cm from liquid nitrogen for 20-30 seconds and when the crystallisation process began, the straws were immersed in a water bath at 20ºC for 10-15 seconds. The vitrification medium was removed rinsing the embryos into a solution containing DPBS with 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS for another 5 min. Only non-damaged embryos (intact mucin coat and pellucid zone) were considered to continue with the transfer, so from all the embryos that were recovered successfully, 101 and 310 from G0 and G1 respectively were catalogued as transferable attending to International Embryo Transfer Society classification (Stringfellow & Seidel, 1998).

2.2.3. Embryo transfer by laparoscopy

A total of 101 (G0) and 310 (G1) warmed embryos by laparoscopy were transferred into the oviducts of 7 (G0) and 20 (G1) healthy and fertile surrogate mothers,

following the procedure previously described by Besenfelder and Brem (1993). The equipment used was a Hopkins® Laparoscope, which is a 5-mm straightviewing laparoscope, 30-cm in length, with a 5-mm working channel (Karl Storz Endoscopia Ibérica S.A. Madrid). To sedate the does during laparoscopy, anesthesia was administered by an intramuscular injection of 5 mg/Kg of xylazine (Bayer AG, Leverkusen, Germany), followed 5-10 min later by an intravenous injection into the marginal ear vein of 6 mg/Kg of ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). During laparoscopy, 3 mg/kg of morphine hydrochloride (Morfina®, B. Braun, Barcelona, Spain) was administered intramuscularly. After transfer, does were treated with antibiotics (4mg/Kg of gentamicine every 24h for 3 days, 10% Ganadexil, Invesa, Barcelona, Spain) and analgesics (buprenorphine hydrochloride (0.03mg/Kg every 12 hours for 3 days, Buprex®, Esteve, Barcelona, Spain) and 0.2mg/Kg of meloxicam every 24h for three days, Metacam® 5mg/mL, Norvet, Barcelona, Spain).

2.3. Embryo survival during gestation and Sperm quality assessment

Embryo survival rate was assessed by calculating the difference between the number of offspring at birth (live or death) and total embryos transferred expressed as percentage.

Routine diagnostic semen analyses were carried out in this experiment. Semen samples were obtained by artificial vagina and collected into a sterile tube. Previously, males began the training period with an artificial vagina at 20 weeks of age (pubertal age, Ewuola & Egbunike, 2010). Training was performed for 4 weeks and then males started the trial (24 weeks of age). For the training and production period, two ejaculates were collected per male and week on a single day using an artificial vagina, with a 30 min interval between collections. Collections from each male were performed on the same day of the week for 11 and 9 weeks in G1 and G2, respectively. Only ejaculates that exhibited white colour were used in the experiment. Samples containing urine and cell debris were dis-carded while gel plugs were removed. An aliquot of sperm sample from each male is stored at -80°C for subsequent epigenetic analysis. Male ejaculates from the same experimental group were weekly pooled as a single sample, evaluating a total number of 11 NC and 11 VT in G1 and 9 NC and 9 VT in G2. The semen quality variables determined were sperm concentration, total sperm count, percent motility,

sperm progressive motility, viability (integrity of the plasma membrane of the head), abnormal sperm (ABN) and normal apical ridge (NAR, acrosomal status). Aliquots from each ejaculate (20µl) were diluted 1:20 with Tris-citrate-glucose extender (250 mM tris-hydroxymethylaminomethane, 83mM citric acid, 50mM glucose, pH 6.8–7.0, 300 mOsmkg-1) to assess total motility and sperm progressive motility using an Integrated Semen Analysis System v. 1.0.17 (ISAS; Projectes i Serveis R+D S.L). The system was set to record images at 30 frames/s. Then, 10 µl of the sample was placed in a 10 µm deep Makler counting chamber. Motility was assessed at 37 °C at 20x using a negative phase contrast microscope. For each sample, four microscopic fields were analysed and a minimum of 400 sperm were evaluated. Before field analysis, we proceeded to identify each sperm trajectory to eliminate debris (false captures) and reduce the risk of confusing trajectories. The same sample was assessed for the percentage of live and dead spermatozoa using the LIVE/DEAD sperm viability kit (Molecular Probes), which consists basically of two DNA-binding fluorescent stains: a membrane-permeant stain, SYBR-14, and a conventional dead-cell stain, propidium iodide (Viudes de Castro et al., 2014). A minimum of 100 sperm cells were counted per ejaculate. Another aliquot was also diluted 1:50 with 0.25% of glutaraldehyde solution in phosphate buffered saline to calculate the concentration in a Thoma- Zeiss counting cell chamber and evaluate both the percentages of NAR and ABN (spermatozoa with morphological abnormalities of head, neck-midpiece and tail) by phase contrast at x400 magnification. The percentage of sperm with NAR was calculated as the ratio: [NAR/(NAR + DAR)] x 100 (damage apical ridge, DAR). The percentage of ABN was calculated as the ratio: [ABN/(ABN + NS)] x 100 (normal sperm, NS).

2.4. Peripheral blood parameters study

At week 56 (late adulthood) when the growth plate is closed (Kilborn et al., 2002), individual blood samples from males were obtained from the central ear artery and dispensed into EDTA-coated tube (Deltalab S.L., Barcelona, Spain). Within 10 minutes of collection, samples were analysed using an automated veterinary haematology analyser MS 4e automated cell counter (MeletSchloesing Laboratories, France) according to the manufacturer's instructions. The blood parameters recorded were white blood cells,

lymphocytes, monocytes, granulocytes, red blood cells and haematocrit. Samples were processed in duplicate.

2.5. Statistical analysis

A generalized linear model (GLM) was applied to semen parameters analyses, with experimental group (VT and NC) and generation (G1 and G2) as fixed effect. The same was done for haematological parameters, and the results have been showed as least squares means ± standard error of means (SEM). Meanwhile, the embryo survival rate was analysed using a probit link with binomial error distribution, including the experimental group (NC and VT) and generation (G1 and G2) as fixed effects, assigning 1 to implanted embryos and offspring born does and 0 to non-implanted embryos and non-born offspring does.

A p-value less than 0.05 was considered to indicate a statistically significant difference. Statistical analyses were carried out using a commercially available software program (SPSS 21.0 software package; SPSS Inc., Chicago, Illinois, USA, 2002) with a post hoc Bonferroni statistic test for GLM and a X^2 of Wald for probit link.

2.6. Epigenetics

2.6.1. Genome-wide DNA methylation profiling

DNA was isolated in the ejaculates used to constitute the following generation on each experimental group (5 NC and 5 VT from each generation). In order to remove no germinal cells (Das et al., 2010), a sperm aliquot (20 µl) containing no more than 10^3 spermatozoa was layered over the top of 1ml HISTOPAQUE®-1077 and centrifuging at 4.500 rpm for 10 min at RT.

The purified sperm was incubated overnight in a lysis solution (SDS 10%, EDTA0.5M, Tris-HCL 1M, NaCl 4M) plus proteinase K (20 ng/ml), DTT 1M and Triton 100X at 50°C. Afterwards, a short 20 min incubation was performed in PureLink RNase A (25mg/ml, Invitrogen, Carlsbad, CA). The DNA was precipitated using isopropanol plus ammonium acetate (10M) and washed with 75% ethanol. Quantification and quality parameters of DNA was carried out using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) spectrophotometer. DNA integrity and concentration were checked on 1% agarose gels and visualized by SYBR® Safe DNA gel stain (10000X, Invitrogen, Carlsbad, CA) in UV lamp.

DNA samples were shipped to Macrogen company (Seoul, South Korea) for methyl-CpG binding domain Sequencing (MBD-Seq). Methylated DNA was obtained using the MethylMiner Methylated DNA Enrichment kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, fragmentation of 1 µg of genomic DNA was performed using adaptive focused acoustic technology (AFA; Covaris) and captured by MBD proteins. The methylated DNA was eluted in high-salt elution buffer. DNA in each eluted fraction was precipitated using glycogen, sodium acetate, and ethanol, and resuspended in DNase-free water. The eluted DNA was used to generate libraries following using TruSeq Nano DNA Library Prep kit (Illumina) standard protocols. The eluted DNA was repaired, an A was ligated to the 3' end, and Illumina adapters were ligated to the fragments. Once ligation was assessed, the adapter-ligated product was PCR amplified. The final purified product was quantified using qPCR Quantification Protocol and qualified using Agilent Technologies 4200 TapeStation (Agilent technologies). The samples were sequenced using the HiSeqTM 4000 platform (Illumina) and paired-end sequencing reads (101 bp) were generated.

Before FastQC (version 0.10.0) quality verification (Andrew, 2010; Zhou et al., 2016), Trimmomatic (version 0.32) was used to eliminate adapters and bases with quality lower than 3 from the end of reads from raw sequence data (Bolger, et al., 2014). In order to map DNA fragments obtained from MBD sequencing, the cleaned reads were aligned to *Oryctolagus cuniculus* (OryCun2.0 Ensembl assembly accession: GCA_000003625.1) reference genome with bowtie2 (version 2.3.4.1) (Langmead & Salzberg, 2012). Mapped data (SAM file format) were performed sorting and indexing using SAMtools.

2.6.2. Data analysis

MEDIPS (version 1.38.0), a computational framework that target the enrichment methylated regions (MRs), was used to perform the MBD data analysis (Lienhard et al., 2013). MEDIPS R package annotates MRs according to the reference genome OryCun2.0 and explore methylation distribution in specified ROI among which we find: promoter, gene, CDS, intron and 3' and 5' UTR. The read coverage of the extended reads was calculated at genome wide 250bp window size and the resulting coverage profiles at each genomic bin were calculated as read count, Reads Per Kilobase Million (RPKM) and

Relative Methylation Score (RMS). Read counts were normalized using TMM normalization to compare the methylation level between NC and VT in both generations. Pair-wise test for extract the DMRs with the negative binomial distributed read counts was performed with exactTest function of edgeR (Robinson, et al., 2010). The significant results are selected on conditions of raw p.value lower than 0.05.

The level of methylation between ROIs was analysed through a GLM with experimental group (VT and NC) or generation (G1 and G2) as fixed effect, depending on what was compared.

ClustVist software was used to generate the PCA and the Heat-Map clustering of DMRs (RPKM as data base; Metsalu & Vilo, 2015). The hierarchical clustering of samples was performed using the "average linkage" as agglomeration method and "Euclidean" as distance metric. InteractiVenn software was used for Venn diagram construction (Heberle et al., 2015).

2.6.3. Functional annotation, KEGG pathways and clustering

The Functional Annotation Tool from Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8; October 2016) (http://david.abcc.ncifcrf.gov/, Huang et al., 2009a, 2009b) a bioinformatic software, was used to study the biological meaning of the genes that belongs to the DMRs. The enriched Gene Ontology™ terms (GO terms) were classified in three GO annotation domains: biological process, cell component and molecular function. Significant enriched GO terms and altered pathways were filtered according to p-value<0.1. Due to the similar, redundant, and heterogeneous annotation contents from the same or different resources, a functional annotation clustering was performed to explore relationships among the annotation terms via the degrees of co-associated genes. The algorithm used tries to group those related genes based on the agreement of sharing similar annotation terms by Kappa statistics. The annotation groups will be ranked based on Enrichment Score and the highest classification stringency was applied.

3. Results

3.1. Embryo survival rate, haematological parameters and sperm traits

Despite the minor number of offspring born in VT group, no significant were observed in the first generation (Table 1; p = 0.214). Nevertheless, in the second generation, the VT group registered a significant lower survival rate (0,71 ± 0,047 vs. 0,34 ± 0,027, NC and VT respectively). The vitrification efficiency registered a decrease from G1 (65%) to G2 (34.2%) after two consecutive embryo vitrification procedures, suggesting a possible accumulative effect.

Table 1. Vitrification survival rate from naturally-conceived rabbits (NC) and vitrified-transferred rabbits (VT) at two generations.

		n	Embryo survival (Mean ± SEM)
G1	**NC**	97	0,73 ± 0,045
	VT	100	0,65 ± 0,048
G2	**NC**	95	0,71 ± 0,047
	VT	310	0,34 ± 0,027*

* indicates significant differences between naturally-conceived and vitrified-transferred (P<0.05). Mean ± SEM: mean ± standard error
n: number of implanted embryos; G1 and G2: generation 1 and 2 respectively

Haematologic parameters revealed no significant differences between experimental groups in both generations, except monocytes, with higher values in VT group at G1 (Table 2). The normal values in New Zealand white rabbit was detailed (Moore et al., 2015).

Table 2. Haematological comparison, assessing the effect of the embryo vitrification.

	G1		G2		Physiological values
	NC	**VT**	**NC**	**VT**	
n	8	10	10	11	
WBC (10^3/mm^3)	9,4 ± 0,91	11,4 ± 0,81	6,6 ± 0,45	7,9 ± 0,43	5.5 – 12.5
RBC (10^6/mm^3)	6,5 ± 0,14	6,5 ± 0,12	7,6 ± 0,26	7,2 ± 0,25	5.46 – 7.94
HTO (%)	45,4 ± 1,19	42,7 ± 1,07	53,1 ± 2,04	48,7 ± 1,95	33 – 50
LYM (%)	41,6 ± 2,68	35,9 ± 2,40	36,3 ± 2,55	34,1± 2,43	28 – 50
MON (%)	16,9 ± 1,09	21,6 ± 0,98*	6,0 ± 0,46	6,4 ± 0,44	4 – 12
GRA (%)	41,4 ± 2,94	42,6 ± 2,63	57,7 ± 3,00	59,9 ± 2,86	38 – 50

* differences between NC and VT from the same generation, statistical differences (p<0.05).
Mean ± SEM: mean ± standard error
n: number of blood samples; NC: naturally-conceived; VT: vitrified-transferred WBC: white blood cells; RBC: red blood cells; HTO: haematocrit; LYM: lymphocytes; MON: monocytes; GRA: granulocytes

Sperm parameters registered similar results, except spermatic concentration at G2, with lower values in the VT group (Table 3). If we compare the two generations inside each experimental group, sperm motility parameters decrease significantly in G2 populations (Table 3).

Table 3. Seminal traits from the two populations from both generations.

	G1		**G2**	
	NC	**VT**	**NC**	**VT**
n	11	11	9	9
SPERM QUALITY PARAMETERS				
CONC (x 10^6 spz/ml)	252,7 ± 27,45	197,5 ± 28,79	436,0 ± 37,54	249,4 ± 37,54*
MOT (%)	89,2 ± 1,99	90,5 ± 1,99	85,9 ± 2,22	82,3 ± 2,22$^+$
PROG (%)	51,5 ± 2,72	53,5 ± 2,72	35,3 ± 2,23$^+$	37,2 ± 2,23$^+$
NAR (%)	95,0 ± 0,76	92,8 ± 0,80$^+$	96,4 ± 0,92	96,5 ± 0,92
ABN (%)	20,5 ± 1,89	17,8 ± 1,99$^+$	24,0 ± 2,86	27,0 ± 2,86
VIAB (%)	89,9 ± 2,32	84,1 ± 2,32	84,9 ± 2,61	84,8 ± 2,61
SPERM MOTILITY PARAMETERS				
VCL (µm/s)	100,5 ± 3,07	102,1 ± 3,07	93,1 ± 2,79	95,3 ± 2,79
VSL (µm/s)	48,0 ± 2,13	50,5 ± 2,13	35,2 ± 1,84$^+$	37,7 ± 1,84$^+$
VAP(µm/s)	69,4 ± 1,66	72,0 ± 1,66	58,3 ± 2,61$^+$	59,7 ± 2,61$^+$
LIN (%)	47,9 ± 2,48	47,9 ± 2,48	37,3 ± 1,38$^+$	39,3 ± 1,38$^+$
STR (%)	69,1 ± 2,28	69,1 ± 2,28	60,0 ± 1,46$^+$	62,9 ± 1,46
WOB (%)	68,8 ± 1,59	68,8 ± 1,59	62,1 ± 1,35$^+$	63,4 ± 1,35$^+$
ALH (µm)	2,4 ± 0,12	2,4 ± 0,12	2,6 ± 0,18	2,6 ± 0,18
BCF (Hz)	9,9 ± 0,53	9,9 ± 0,53	8,0 ± 0,31$^+$	9,0 ± 0,31

* indicates statistical differences (P<0.05) between experimental groups

$^+$ indicates statistical differences (P<0.05) between generations in each experimental group

Mean ± SEM: mean ± standard error

n: number of pools of ejaculates analysed; G1 and G2: generation 1 and 2 respectively; NC: natural conceived animals; VT: vitrificated-transferred animals; CONC: Spermatic concentration; MOT: Percentage of sperm motility; PROG: Percentage of progressive motility; NAR: percentage of normal apical ridge; ABN: Percentage of abnormal forms; VIAB: Percentage of viable sperm; VCL: Curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity index; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat cross-frequency.

3.2 MDB analysis

In order to study the sperm pattern methylation in rabbit, MBD sequencing was performed on both experimental groups (NC and VT) and generations (G1 and G2). After quality verification and trimming procedure, about 19 million raw sequence reads per sample were generated. Around 80% of the reads mapped to the OryCun2.0 reference genome, and from those close to 90% mapped uniquely. The multiple mapped read were removed from the analyses (Table 4).

Table 4. Mapping data generated by MBD-sequencing.

		n	Number of raw sequence reads	Percentage of mapped reads in total reads (%)	Percentage of uniquely mapped reads (%)
G1	**NC**	5	19,093,900.8	79.57	87.45
	VT	5	19,551,377	78.94	86.65
G2	**NC**	5	19,309,490.6	78.28	88.07
	VT	5	19,862,202	79.64	87.44

n: number of samples; G1 and G2: generation 1 and 2 respectively; NC: naturally-conceived animals; VT: vitrificated-transferred animals

3.2.1 Sperm methylation distribution

With the aim to study the distribution of the methylation through the rabbit genome at sperm level, the following region of interest (ROI) were stablished: promotor, 5 and 3 UTR, CDS and Intron. Afterwards, it was evaluated the level of methylation in each ROI based on the relative methylation score (Lienhard et al., 2014; Figure 2). The most methylated regions were at CDS, intron and 3'UTR, meanwhile promoter and 5'UTR showed a minor score. On the other hand, if the level of methylation is compared between generations (VT_G1 vs VT_G2; NC_G1 vs NC_G2), in all ROIs, the animals with two successive embryo vitrifications (VT_G2) showed a higher methylation level respect to animals that have been submitted to only one vitrification (VT_G1; Figure 2).

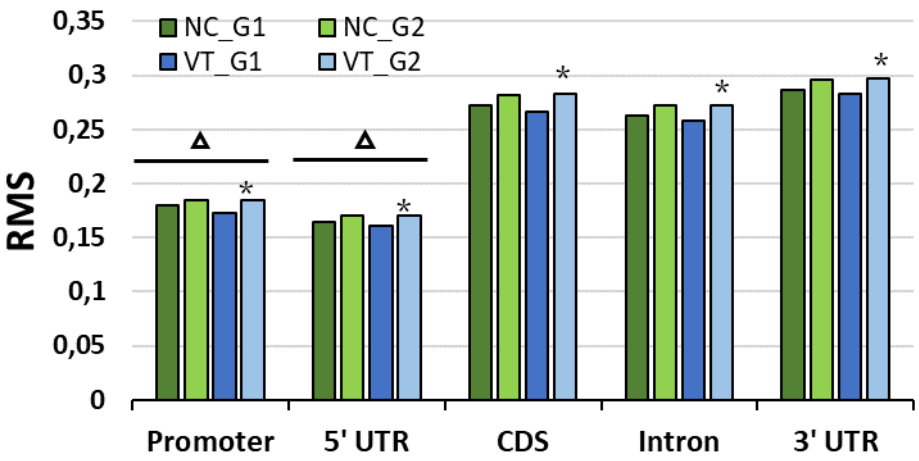

Figure 2. Global methylation distribution in the different ROIs between experimental groups and generations. Significant differences (p.value < 0.05) were shown as a triangle for the differences between ROI and as asterisks for the differences between generations in each ROI.

3.2.2 Differential methylation analyses

After an EdgeR analysis (Robinson, et al., 2010), the number of DMR in each ROI were identified (Supplementary data 1). The statistical analysis of RMS from DMR in each ROI and generation can be observed in the Figure 2. In this case, only the promoter registered a significant lower RMS (Figure 3). In this case, the level of methylation is compared between experimental group from each generation (NC_G1 vs VT_G1; NC_G2 vs VT_G2), in all ROIs. Animals from the G1 presented significant differences (p>0.05), specifically VT animals (VT_G1) that showed a lower methylation score respect to the NC animals (NC_G1) in the body gene ROIs (Figure 3). Meanwhile, in the promoter VT animals from both generations showed a significant increase respect to the NC animals.

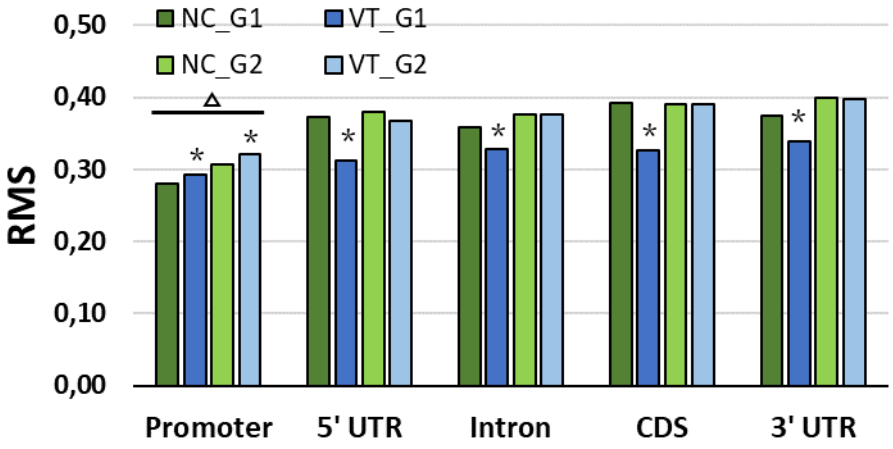

Figure 3. RMS in the different ROIs in both groups and generations based on DMR. Significant differences (p.value < 0.05) were shown as a triangle for the differences between ROI and as asterisks for the differences between experimental groups in each ROI.

A PCA and HM of the samples based on DMR (RPKM) separates both experimental groups, without big differences between generations (Figure 4).

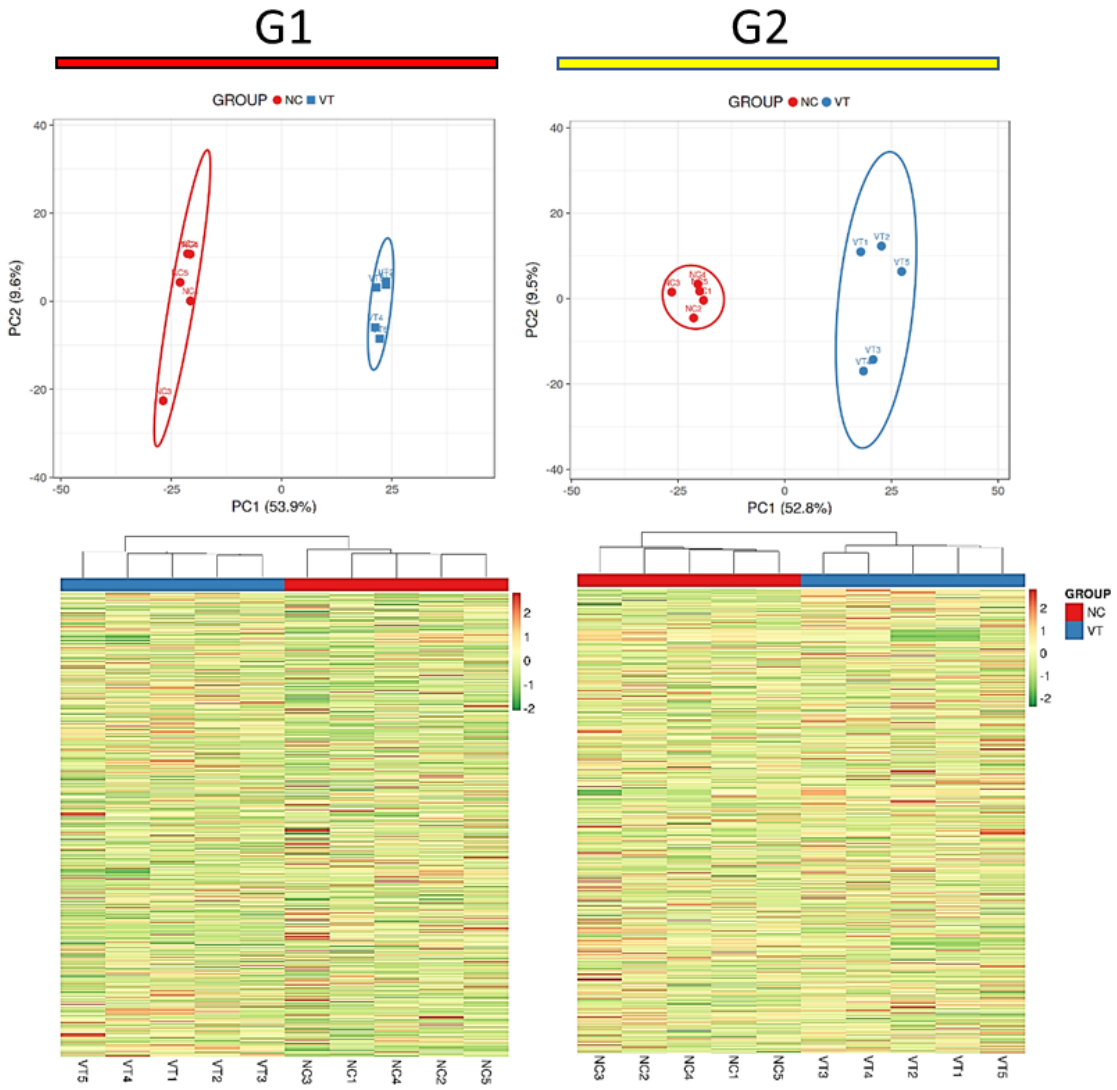

Figure 3. Distribution of the two experimental groups, vitrified-transferred animals (VT) and naturally-conceived (NC), which was compared in each generation (G1, G2). Top: Principal Component Analysis (PCA). Bottom: Heat-Map clustering (HM).

3.2.2 Functional annotation

The functional analysis was performed using the DMR in Gene and Promoter ROIs. The sum of both ROIs provided 1049 DMRs from G1 and 987 DMRs from G2. From those, we were able to identify 809 (G1) and 727 (G2) differentially methylated genes (Supplementary data 2). On the other hand, if the differentially functional analysis is performed taking in consideration only the hyper- or hypomethylated genes (VT/NC ratio), and, it is compared to the functional analysis including both hyper- and

hypomethylated genes (Supplementary data 3), most the coincidences were found in the hypermethylated genes.

The enriched GO terms were classified in three different domains: biological processes, molecular function and cellular compartment. Among the GO term affected translation, ribosome and structural constituent of ribosome are the most altered, having similar gene members that are hypo and hypermethylated (VT/NC ratio) in both generations. Between generations, 3 common KEGG pathways: ribosome, alzheimer's disease and parkinson's disease a functional annotation was registered (Table 5).

Moreover, a cluster analysis was carried out. This analysis is used to explore relationships among the annotation terms provided by DAVID software. Among the 10 Functional Annotation Cluster in G1 and the 7 in G2 (Supplementary data 4), a cluster compose with mannose-binding lectin, zymogen granule membrane (CC GO term) and carbohydrate binding (MF GO term), which genes are hypomethylated in VT group, presented the highest enrichment score in both generations.

Table 5. Functional annotation table with the Gene Ontology (GO) terms and KEGG pathways that are enrichment. Were obtained from differentially methylated genes (DMGs) from promoter and gene in the two generations and from the common DMGs between the two generations.

	G1	p-value	G2	p-value	COMMON	p-value
BP	Translation	2,30e-09	Translation	3,50e-05	Translation	1,30E-04
	Ossification involved in bone maturation	6,60e-02	Ossification involved in bone maturation	1,00e+00	Ossification involved in bone maturation	2,20E-02
	Phagocytosis, recognition	5,30e-02	Acylglycerol catabolic process	9,90e-01	Muscle contraction	9,70E-02
	Protein localization to photoreceptor Outer segment	9,10e-02	Negative regulation of cardiac muscle cell apoptotic process	9,90e-01	Skeletal system morphogenesis	9,70E-02
	Ribosomal small subunit biogenesis	9,10e-02	Dna repair	9,80e-01		
	Small gtpase mediated signal Transduction	7,90E-02				
CC	Ribosome	1,10e-05	Ribosome	1,00e-03	Ribosome	1,80E-03
	Zymogen granule membrane	5,30e-06	Zymogen granule membrane	6,60e-02	Zymogen granule membrane	6,40E-05
	Integral component of membrane	9,20e-02	Membrane	9,70e-01	Membrane	1,50E-02
	Voltage-gated calcium channel complex	2,60e-02			I band	5,10E-02
					T-tubule	7,30E-02
					Voltage-gated calcium channel complex	7,30E-02
					Smooth endoplasmic reticulum	4,20E-02
MF	Structural constituent of ribosome	2,20e-08	Structural constituent of ribosome	3,20e-05	Structural constituent of ribosome	3,30E-04
	Carbohydrate binding	4,90e-03	Carbohydrate binding	3,50e-01	Carbohydrate binding	5,80E-03
	Olfactory receptor activity	1,10e-05	Heme binding	2,30e-01	Cytochrome-c oxidase activity	9,70E-02
	G-protein coupled receptor activity	6,20E-05	Oxidoreductase activity	4,40E-01		
	Voltage-gated calcium channel activity	5,60e-02	Acylglycerol lipase activity	6,50e-01		
	Calmodulin binding	7,80e-02	Iron ion binding	7,30e-01		
	Damaged DNA binding	8,80E-02	G-protein coupled receptor activity	7,80E-01		
	Pheromone receptor activity	9,30e-02	Olfactory receptor activity	5,90e-01		
			Lyase activity	7,10E-01		
			Double-stranded RNA binding	7,20E-01		
KEGG	Ribosome	1,30E-05	Ribosome	9,80E-04	Ribosome	2,00E-05
	Alzheimer's disease	4,10E-02	Alzheimer's disease	7,40E-01	Alzheimer's disease	1,00E-02
	Parkinson's disease	5,20E-02	Parkinson's disease	9,70E-01	Parkinson's disease	2,70E-02
	Vascular smooth muscle contraction	4,90E-02			Pancreatic secretion	5,30E-02
	Oxidative phosphorylation	8,30E-02			Huntington's disease	6,20E-02
	Olfactory transduction	9,30E-05			Vascular smooth muscle contraction	6,00E-02
					Oxidative phosphorylation	8,30E-02

G1 and G2: generation 1 and 2 respectively; BP: biological process; CC: cellular components; MF: molecular functions
Red: hypermethylated; **Blue**: hypomethylated; **Green**: hyper and hypomethylated

3.2.3 Common differentially methylated genes

From those DMRs used for the functional analyses, it was evaluated if the DMR obtained in G1 were maintained differentially methylated in G2. A total of 387 DMRs remained differentially methylated (around 40%; Figure 5). From the 387 DMRs, 306 DMGs were identified. The functional analysis was similar to the G1 and G2 analysis including the non-common genes (Supplementary data 5), likely, due to the large amount of genes in common between generations.

From the 306 DMRs, 187 were hypermethylated and 119 hypomethylated (VT/NC ratio) (Supplementary data 2). The comparison between hypo/hypermethylated and global functional analysis revealed that most of terms were hypermethylated (Supplementary data 5). Also, a cluster analysis was carried out with the common DMGs and only 5 were detected within which is detected the cluster with mannose-binding lectin, zymogen granule membrane and carbohydrate binding (Supplementary data 6).

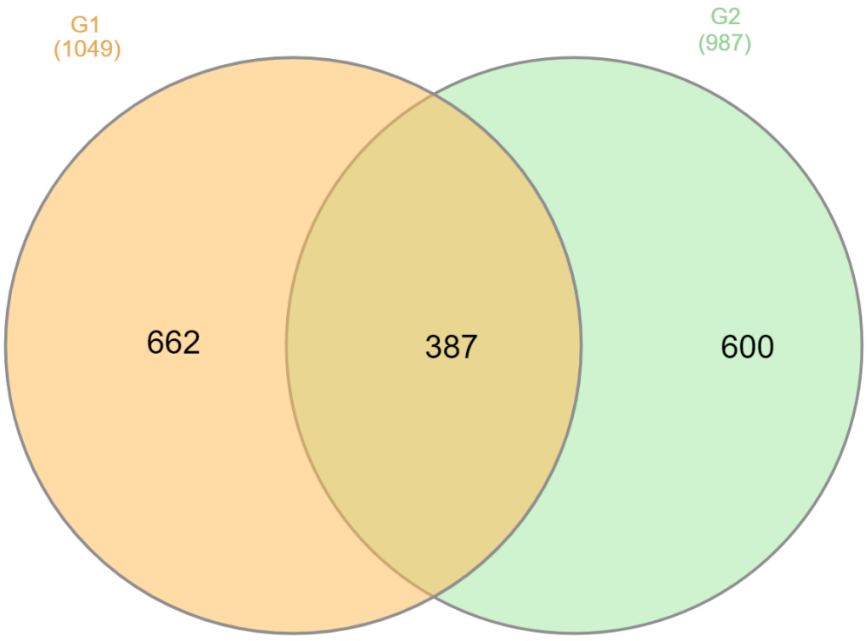

Figure 4. InteractiVenn between the total DMRs detected in G1 and in G2. 387 DMRs were detected in both generations, meanwhile 662 were exclusive of G1 and 600 of G2.4. Discussion

4.1. Survival rate, seminal parameters and haematological analyses

The negative effect of early embryo cryopreservation on survival rate registered in the current study is in concordance with previous studies in this species. In fact, the embryo rabbit cryopreservation has induced higher embryo losses (Saenz-de-Juano et al., 2012, 2014b) or alterations in foetal survival or development (Mocé et al., 2010; Marco-Jiménez et al., 2013; Vicente et al., 2013). These alterations has been not limited to pre-natal stages, but also at postnatal phases, with a differential phenotype (Lavara et al., 2015; Garcia-Dominguez et al., 2020a). These postnatal modifications may be originated from pre-natal alterations (Saenz-De-Juano et al., 2014a, 2015) or as a consequence of phenotype plasticity phenomena (Forsman 2015; Garcia-Dominguez et al., 2020c). Anyhow, all the results obtained in rabbit indicates that the embryo cryopreservation is not a neutral process, and this hypothesis is confirmed by the negative alterations at pre- and postnatal level have been also reported in other livestock species (Ghosh et al., 2017; Gupta et al., 2017; Siqueira et al., 2020).

Further the direct vitrification effect, in the current study, it was analysed the effect of successive applications of a cryopreservation program. This second round may be inducing a negative accumulation, being having registered a 34%. of survival rate decrement from G1 to G2. The negative effects of embryo vitrification reported on rabbit foetal survival and development (Mocé et al., 2010; Marco-Jiménez et al., 2013; Vicente et al., 2013) may persist throughout the offspring life-span (Canovas et al., 2017). In fact, a minor weight, growth rate and organ weight were detected in adult rabbits originated from vitrified embryos (Lavara et al., 2015; Garcia-Dominguez, et al., 2020a, 2020b).

Vitrification is done in preimplantation embryos during epigenetic reprogramming period (Duranthon et al., 2008; Lou et al., 2018), consequently the epigenetic marks may be affected by cryopreservation process. The modifications registered in adult weight, growth rate or organ weight at postnatal stage indicate a possible modification of the epigenetic marks at not only embryo or foetal stage, but also adult stage. In fact, an altered adult liver transcriptome and proteome in the offspring from embryo vitrified parents was reported (Garcia-Dominguez, el al., 2020b, 2020c). Therefore, it is possible that not only the somatic cells were affected by ARTs,

but also the germinal line (intergenerational effect). Similar results have been observed in other species (Calle et al., 2012; Illum et al., 2018) when cattle offspring inherit the metabolic or abnormal phenotype from them parents which were undergone to an *in vitro* fertilization and culture. These results may be indicating a possible transmission of altered epigenetic profiles via germ line to subsequent generations leaving a parental genetic imprint (Rodrígez & Sánchez, 2019; Garcia-Dominguez et al., 2020c).

Developmental Origins of Heath and Disease (DoHaD) hypothesis postulates that exposure to stressful environmental in embryo early development can impact the health of offspring (Feuer & Rianudo, 2016). In our study, all the descendants who were born were healthy, supported by the haematological analysis of the peripheral blood, which not differ from naturally-conceived animals. The possible significant differences in some haematological parameters from the physiological values could be related to the low number of samples. The differences in sperm quality in G2 respect to G1 may be due to an external factor. In humans, it has not been possible to correlate bad quality sperm to the appearance of adverse phenotypes after undergoing ART procedures (Whitcomb et al., 2017; Pastuszak et al., 2019). Additionally, the ejaculates from vitrified-transferred experimental group did not show reproductive effectiveness differences respect to NC group. This lack of differences may be because selection plays in favour to individuals or flexible genotypes that can survive modifying their growth, development and behaviour, described within evolutionary biology as phenotypic plasticity (Forsman, 2015).

4.2. Epigenome analysis

Several evidences indicate that the adult phenotype could be affected by the embryo environment, and these modifications could be caused by altered DNA methylation patterns (Ghosh et al., 2015; Salilew-Wondim et al., 2018). The mature sperm has the ability to reveal some of these subtle patterns because has the potential to retain epigenetic variations, as it has been reported when sperm is compared to the oocyte in pig (*Sus scrofa*) and cow (Ivanova et al., 2020). Therefore, modification in the sperm methylation can be have an effect on subsequent generations (Jenkins et al., 2017; Rodríguez & Sánchez, 2019). Nevertheless, it is the first time that it is described the effect of a successive vitrification process on germinal line.

Considering the global methylation density across gene regions, the level of MRs in gene body are higher than the promoter that remains less methylated. Similar results have been shown in human sperm exposed to Bisphenol A, an endocrine disrupting (Zheng, et al., 2017), or in bovine placenta after *in vitro* embryo manipulation (Su, et al., 2014). Same pattern was revealed when it is evaluated the methylation distribution based on DMRs. In pig and cow sperm, the DMR analyses reported similar results under *in vitro* production and maturation (Ivanova, et la., 2020). If the level of methylation between generations is compared (based on RMS), a higher level was observed in G2 respect to G1. These results are in concordance with the hypothesis of accumulative effect of embryo cryopreservation process.

4.3. Functional Analysis of the Differentially Methylated Genes

Epigenetic differences between experimental groups have been registered in both generations, which may be contributing in the reproductive alterations observed. In order to know which physiological process could be affected, a functional analysis was performed. From all GO terms and KEGG pathway significantly altered, a relevant amount was in relation to protein metabolism (translation in biological process, ribosome in cellular components, structural constituent of ribosome in molecular functions and ribosome in KEGG pathways). The abundance hypermethylated genes in the VT group related to tRNA and ribosomal proteins, that provide the apparatus for protein synthesis, may be causing alterations in the novo protein synthesis and consequently modifying the cell functionality and thereby early embryonic development (Palasin, et al., 2019). According to our findings, some studies in bovine demonstrate similar alterations in embryo protein metabolism under *in vitro* manipulation (Corcoran et al., 2006), also under a major stress factor such as cryopreservation (Aksu, et al., 2012).

DMGs in relation to oxidative phosphorylation (OXPHO) were also found. OXPHO is characteristic of mitochondria's activity, and the genes registered in relation to were hypermethylated in the VT group, indicating a possible modification of the energy availability in sperm cells. Studies with bovine embryos under in vitro production and maturation promote mitochondrial dysfunction and systemic oxidative stress (Feuer et al., 2014), and reveals that a total repression of energy production via OXPHO may incur

in a normal embryo development (Thompson et al., 2000). Additionally, mitochondrial OXPHO is the primary source of ATP to support life and development (Garcia-Dominguez et al., 2020b), and the lack of ATP may be related to diseases such as Alzheimer's disease and Parkinson's disease (Tachibana et al., 2018) that appears between our KEGG pathways altered. Nevertheless, in other studies no alteration of energy levels were found in mouse and bovine embryos (Amoushahi et al., 2013; Gaviria et al., 2018) after cryopreservation. The reason could be that in these cases, a previous embryo in vitro culture with resveratrol was applied.

Carbohydrate-binding GO term appeared hypomethylated in the VT group. This GO term is in relation to the recognition of carbohydrate components on the embryo zona pellucida and mediate the sperm-oocyte adhesion (Clark, 2013). The cluster analyses revealed a relation with mannose-binding lectin localized in the apical region of the sperm head being able to get involved in the sperm-oocyte adhesion process (Zigo et al., 2020). A highly expression of the genes that compose this function increase the effectiveness in the fecundation. Nevertheless, no difference in fertility rate were found between experimental groups.

A cluster affected the olfactory receptor activity is significant hypomethylated in the VT group. Olfactory receptor neurons are associated with molecular recognition and sensory transduction that involve the membrane and the voltage-gated channels (Getchell, 1986). Epigenetic mechanism could be relevant to the regulation of neural development and a highly expression is necessary for a neuron differentiation through successive stages of development (McDonald et al., 2010). Same GO term appears in many other studies that analyse the modifications in DNA in bovine and horses (Huang et al., 2018; Zi et al., 2018). But in that case embryo is under many other TRAs that shows to affect DNA methylation pattern.

Notwithstanding, as said Mani et al. (2020), is difficult to find the relationship between aberrant epigenetic marks and adverse perinatal outcomes and it is unclear whether of that will have long-term effects on health and disease, because ART procedures affect PGCs.

4.4. Common DMGs reveals a repetitive pattern

A total of 387 DMRs were common between both generations. It is known that prior fecundation, sperm is actively and passively demethylated to increase the gene activity, and then remethylated (Farthing, et al., 2008; Messerschmidt, et al., 2014). However, the amount of DMRs that were in common between generations, agreeing with the fact that in many cases the gestational exposure to an environmental stress promotes the male genome to scape this reprogramming event in preimplantation embryos, and as a result the embryo maintain the sperm methylation pattern allowing being heritable (Branco, et al., 2008; de Barros & Paula-Lopes, 2018; Illum et al., 2018; Carrel, 2019).

Regarding the functional annotation of common DMGs, there are many similar enrichments GO and altered pathways to what we see on the G1 and G2. The most of them were hypermethylated, revealing that the epigenetic marks promoted by cryopreservation procedure were retained. That could be explain because there are possible areas of the genome that are more sensible or exposed to that chemical changes that the vitrification procedure is responsible. If we maintain the pressure this pattern of alterations could be repeated and many altered metabolism pathways will be expressed at embryo, foetal and postnatal stages and will be responsible of changes that could be detected at long-term.

5. Conclusion

To sum up, the successive application of an embryo cryopreservation process may be contributing in the decrease of embryo survival rate, but not incur in the sperm traits or health of the animals. A big set of the DMRs remained from the G1 to G2 generation, suggesting that the cryopreservation is affecting the germinal line, revealing a possible intergenerational transmission of epigenetic marks. These evidences demonstrated that germplasm preservation programs might not be ensuring that offspring show the expected profile since epigenetic marks may be the responsible of the alterations at embryonic, foetal and postnatal stages.

6. Supplementary materials

Supplementary data 1. Excel document with total DMRs from each ROI in the two generations.

Supplementary data 2. Excel document with DMRs obtained from promoter and gene in the two generations and DMRs that are in common between the two generations. **Red:** Transcript_ID and Gene_ID hypermethylated; **Blue:** Transcript_ID and Gene_ID hypomethylated

Supplementary data 3. Differentially functional annotation analysis comparing the hyper- and hypomethylated genes with the total DMRs from G1 and G2. Table contain the Gene Ontology (GO) terms and KEGG pathways that are enrichment.

G1 and G2: generation 1 and 2 respectively; BP: biological process; CC: cellular components; MF: molecular functions
Red: hypermethylated; **Blue**: hypomethylated; **Green**: hyper and hypomethylated

	G1 TOTAL	p.value	HYPERMETHYLATED	p.value	HYPOMETHYLATED	p.value
BP	Translation	2,30e-09	Translation	7,40e-07	Translation	4,40E-03
	Ossification involved in bone maturation	6,60e-02	Ossification involved in bone maturation	3,60e-02	Ribosomal small subunit biogenesis	4,10E-02
	Protein localization to photoreceptor outer segment	5,30E-02	Protein localization to photoreceptor outer segment	2,90E-02	TOR signaling	9,20E-02
	Phagocytosis, recognition	9,10e-02	Phagocytosis, recognition	5,10e-02		
	Ribosomal small subunit biogenesis	9,10e-02				
	Small gtpase mediated signal transduction	7,90E-02				
CC	Ribosome	1,10e-05	Ribosome	2,80e-04	Ribosome	5,60E-02
	Zymogen granule membrane	5,30e-06	Membrane	5,40e-02	Zymogen granule membrane	2,90E-04
	Integral component of membrane	9,20e-02	I band	8,60e-02	Integral component of membrane	9,70E-03
	Voltage-gated calcium channel complex	2,60e-02	Replication fork	7,90e-02	Plasma membrane	1,70E-04
MF	Structural constituent of ribosome	2,20e-08	Structural constituent of ribosome	3,10e-06	Structural constituent of ribosome	8,80E-03
	Olfactory receptor activity	1,10e-05	Fatty-acyl-coa binding	8,40e-02	Olfactory receptor activity	1,10E-10
	G-protein coupled receptor activity	6,20E-05	Heme binding	9,30E-02	G-protein coupled receptor activity	1,20E-09
	Carbohydrate binding	4,90e-03			Carbohydrate binding	1,30E-02
	Voltage-gated calcium channel activity	5,60e-02				
	Calmodulin binding	7,80e-02				
	Damaged DNA binding	8,80E-02				
	Pheromone receptor activity	9,30e-02				
KEGG	Ribosome	1,30E-05	Ribosome	3,90E-06	Olfactory transduction	1,70E-11
	Alzheimer's disease	4,10E-02	Alzheimer's disease	1,60E-02	Fanconi anemia pathway	9,90E-02
	Parkinson's disease	5,20E-02	Parkinson's disease	8,20E-03		
	Vascular smooth muscle contraction	4,90E-02	Vascular smooth muscle contraction	4,90E-02		
	Oxidative phosphorylation	8,30E-02	Oxidative phosphorylation	7,30E-02		
	Olfactory transduction	9,30E-05	Arachidonic acid metabolism	2,00E-02		
			Pancreatic secretion	9,90E-03		
			Huntington's disease	8,10E-02		
			Non-alcoholic fatty liver disease (NAFLD)	4,00E-02		
			Steroid hormone biosynthesis	7,70E-02		

	G2 TOTAL	p.value	HYPERMETHYLATED	p.value	HYPOMETHYLATED	p.value
BP	Translation	3,50e-05	Translation	4,70e-03	Translation	2,50E-05
	Ossification involved in bone maturation	1,00e+00	Ossification involved in bone maturation	3,00e-02	Negative regulation of protein kinase activity	8,90E-02
	Acylglycerol catabolic process	9,90e-01	Acylglycerol catabolic process	1,80e-02		
	DNA repair	9,80E-01	T cell proliferation	5,90E-02		
	Negative regulation of cardiac muscle cell apoptotic process	9,90e-01				
CC	Ribosome	1,00e-03	Ribosome	6,30e-03	Ribosome	3,10E-03
	Zymogen granule membrane	6,60e-02	I band	7,10e-02	Zymogen granule membrane	3,70E-03
	Membrane	9,70e-01				
MF	Structural constituent of ribosome	3,20e-05	Structural constituent of ribosome	5,10e-03	Structural constituent of ribosome	7,70E-05
	Heme binding	2,30e-01	Heme binding	7,50e-03	Carbohydrate binding	7,80E-03
	Oxidoreductase activity	4,40e-01	Oxidoreductase activity	5,00e-03		
	Acylglycerol lipase activity	6,50e-01	Acylglycerol lipase activity	2,50e-02		
	Iron ion binding	7,30e-01	Iron ion binding	5,10e-02		
	Carbohydrate binding	3,50e-01				
	G-protein coupled receptor activity	7,80E-01				
	Olfactory receptor activity	5,90e-01				
	Lyase activity	7,10e-01				
	Double-stranded RNA binding	7,20E-01				
KEGG	Ribosome	9,80E-04	Ribosome	1,20E-03	Ribosome	6,30E-03
	Alzheimer's disease	7,40E-01	Alzheimer's disease	3,50E-02		
	Parkinson's disease	9,70E-01	Parkinson's disease	7,20E-02		
			Cardiac muscle contraction	4,70E-02		
			Steroid hormone biosynthesis	5,90E-02		
			Non-alcoholic fatty liver disease (NAFLD)	8,70E-02		

Supplementary data 4. Cluster analysis with the DMGs in both generations.

Annotation Cluster G1		Term	p.value
1 ES: 4.91	SMART	SM00915	7.1E-7
	Interpro	Mannose-binding lectin	1.2E-6
	GO Term CC	Zymogen granule membrane	5.3E-6
	GO Term MF	Carbohydrate binding	4.9E-3
2 ES: 4.86	GO Term MF	olfactory receptor activity	1.1E-5
	Interpro	Olfactory receptor	1.3E-5
	Keyword	Olfaction	1.8E-5
3 ES: 0.96	GO Term MF	voltage-gated calcium channel activity	5.6E-2
	Keyword	Calcium channel	6.2E-2
	Keyword	Calcium transport	9.5E-2
	Interpro	Ion transport domain	4.2E-1
4 ES: 0.91	Keyword	Heme	7.3E-2
	GO Term MF	heme binding	1.4E-1
	Keyword	Iron	1.9E-1
5 ES: 0.36	Interpro	Peptidase S1, trypsin family, active site	3.4E-1
	Keyword	Serine protease	3.6E-1
	SMART	Tryp_SPc	3.6E-1
	Interpro	Peptidase S1A, chymotrypsin-type	3.8E-1
	Interpro	Peptidase S1	4.2E-1
	Interpro	Trypsin-like cysteine/serine peptidase domain	4.5E-1
	GO Term MF	serine-type endopeptidase activity	5.9E-1
	KEGG Pathway	Protein digestion and absorption	6.8E-1
6 ES: 0.27	KEGG Pathway	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4.4E-1
	KEGG Pathway	Hypertrophic cardiomyopathy (HCM)	5.7E-1
	KEGG Pathway	Dilated cardiomyopathy	6.1E-1
7 ES: 0.26	SMART	HOX	4.6E-1
	Keyword	Homeobox	5.3E-1
	Interpro	Homeodomain	5.4E-1
	Interpro	Homeodomain-like	7.1E-1
8 ES: 0.21	KEGG Pathway	Aldosterone synthesis and secretion	4.9E-1
	KEGG Pathway	Insulin secretion	5.4E-1
	KEGG Pathway	GnRH signaling pathway	5.5E-1
	KEGG Pathway	MAPK signaling pathway	9.7E-1
9 ES: 0.09	INTERPRO	Zinc finger C2H2-type/integrase DNA-binding domain	7.5E-1
	SMART	ZnF_C2H2	7.7E-1
	Interpro	Zinc finger, C2H2-like	8.3E-1
	Interpro	Zinc finger, C2H2	8.7E-1
10 ES: 0.07	SMART	RING	7.4E-1
	Interpro	Zinc finger, RING-type	8.7E-1
	Interpro	Zinc finger, RING/FYVE/PHD-type	9.8E-1

ES: Enrichment Score. **Blue**: cluster with gene hypomethylated; Red: cluster with gene hyper-methylated

Annotation Cluster G2	Term		p.value
1 ES: 3.31	SMART	SM00915	2.3E-4
	Interpro	Mannose-binding lectin	4.7E-4
	GO Term CC	zymogen granule membrane	1.1E-3
2 ES: 1.37	GO Term MF	olfactory receptor activity	3.4E-2
	Interpro	Olfactory receptor	3.5E-2
	Keyword	Olfaction	4.7E-2
	Keyword	Sensory transduction	6.0E-2
3 ES: 1.11	Interpro	Cytochrome P450, E-class, group I, CYP2D-like	1.5E-3
	GO Term MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	1.8E-2
	Interpro	Cytochrome P450, E-class, group I	1.3E-1
	Interpro	Cytochrome P450, conserved site	1.5E-1
	Interpro	Cytochrome P450	1.7E-1
	Keyword	Monooxygenase	2.3E-1
	Keyword	Oxidoreductase	8.0E-1
4 ES: 1.11	Interpro	G protein-coupled receptor, rhodopsin-like	5.1E-2
	Keyword	G-protein coupled receptor	8.7E-2
	Keyword	Transducer	1.1E-1
5 ES: 0.16	KEGG Pathway	Small cell lung cancer	4.6E-1
	KEGG Pathway	Influenza A	8.2E-1
	KEGG Pathway	Herpes simplex infection	8.7E-1
6 ES: 0.06	SMART	IG	7.4E-1
	Interpro	Immunoglobulin subtype	8.4E-1
	Interpro	Immunoglobulin-like domain	9.3E-1
	Interpro	Immunoglobulin-like fold	9.9E-1
7 ES: 0.04	SMART	S_TKc	7.9E-1
	Interpro	Protein kinase, catalytic domain	9.7E-1
	Interpro	Protein kinase-like domain	9.8E-1

ES: Enrichment Score. **Blue**: cluster with gene hypomethylated; Red: cluster with gene hyper-methylated

Supplementary data 5. Differentially functional annotation analysis comparing the hyper- and hypomethylated genes with the common DMRs.

Table contain the Gene Ontology (GO) terms and KEGG pathways that are enrichment.

	COMMON		HYPERMETHYLATED		HYPOMETHYLATED	
BP	Translation	1,30e-04	Translation	2,10e-04		
	Ossification involved in bone maturation	2,20e-02	Ossification involved in bone maturation	1,50e-02		
	Muscle contraction	9,70e-02	Muscle contraction	6,90e-02		
	Skeletal system morphogenesis	9,70e-02				
CC	Ribosome	1,80e-03	Ribosome	4,20e-04	Zymogen granule membrane	2,60e-04
	Membrane	1,50e-02	Membrane	2,10e-02		
	I band	5,10E-02	I band	3,50e-02		
	T-tubule	7,30E-02	T-tubule	5,00e-02		
	Zymogen granule membrane	6,40e-05				
	Voltage-gated calcium channel complex	7,30e-02				
	Smooth endoplasmic reticulum	4,20e-02				
MF	Structural constituent of ribosome	3,30e-04	Structural constituent of ribosome	4,20e-04	Carbohydrate binding	5,10e-03
	Cytochrome-c oxidase activity	9,70e-02	Cytochrome-c oxidase activity	6,70e-02		
	Carbohydrate binding	5,80e-03	Voltage-gated calcium channel activity	8,40e-02		
KEGG	Ribosome	2,00E-05	Ribosome	1,50E-05		
	Alzheimer's disease	1,00E-02	Alzheimer's disease	1,60E-02		
	Parkinson's disease	2,70E-02	Parkinson's disease	5,20E-02		
	Pancreatic secretion	5,30E-02	Pancreatic secretion	2,50E-02		
	Huntington's disease	6,20E-02	Huntington's disease	9,90E-02		
	Vascular smooth muscle contraction	6,00E-02	Vascular smooth muscle contraction	2,80E-02		
	Oxidative phosphorylation	8,30E-02	Cardiac muscle contraction	7,00E-02		

G1 and G2: generation 1 and 2 respectively; BP: biological process; CC: cellular components; MF: molecular functions
Red: hypermethylated; **Blue**: hypomethylated; **Green**: hyper and hypomethylated

Supplementary data 6. Cluster analysis with the common DMGs.

Annotation Cluster	Term		p.value
1 ES: 3.92	SMART	SM00915	1.8E-5
	INTERPRO	Mannose-binding lectin	3.2E-5
	GO Term CC	zymogen granule membrane	6.4E-5
	GO Term MF	carbohydrate binding	5.8E-3
2 ES: 0.98	UP_SEQ_FEATURE	topological domain:Cytoplasmic	7.0E-2
	UP_SEQ_FEATURE	transmembrane region	1.2E-1
	UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	1.4E-1
3 ES: 0.34	SMART	IG	3.0E-1
	Interpro	Immunoglobulin subtype	3.8E-1
	Interpro	Immunoglobulin-like domain	5.2E-1
	Interpro	Immunoglobulin-like fold	7.1E-1
4 ES: 0.19	GO Term MF	G-protein coupled receptor activity	5.8E-1
	Interpro	G protein-coupled receptor, rhodopsin-like	6.0E-1
	Keywords	G-protein coupled receptor	6.9E-1
	Keywords	Transducer	7.1E-1
5 ES: 0.17	INTERPRO	Olfactory receptor	5.8E-1
	GO Term MF	olfactory receptor activity	6.3E-1
	Keywords	Olfaction	6.3E-1
	Keywords	Sensory transduction	6.6E-1
	Keywords	Cell membrane	9.1E-1

ES: Enrichment Score. **Blue**: cluster with gene hypomethylated; **Red**: cluster with gene hyper-methylated

7. Abbreviations

ABN	Abnormal sperm form
AI	Artificial Insemination
ARTs	Assisted Reproductive Technologies
DMG	Differentially Methylated Gene
DMR	Differentially Methylated Region
DOHaD	Developmental Origins of Health and Disease
ET	Embryo Transfer
G1	First Generation
G2	Second Generation
GLM	Generalized Linear Model
GO	Gene Ontology
HM	Heat-Map
KEGG	Kyoto Encyclopedia of Genes and Genomes
NAR	Normal Apical Ridge
NC	Naturally-Conceived
OXPHO	Oxidative phosphorylation
PCA	Principal component analysis
RMS	Relative Methylation Score
ROI	Region Of Interest
RPKM	Reads Per Kilobase Million
VT	Vitrified-Transferred

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