

Harvesting of Mouse Embryos at 0.5 Dpc as a Tool to Reduce Animal Use: Data from C57BL/6J, B6*129 and FVB/NJ Strains

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Abstract

Superovulation is used to stimulate the production and release of large amounts of oocytes in mice by using two hormones that mimic FSH (PMSG) and LH (hCG) effects. Since superovulation can have a negative impact on oocyte and embryo development, this investigation aimed to compare two alternatives for 2-cells embryo collection in order to reduce the number of females and to benefit from the superovulation process. Data from mouse embryo collection from our facility was analyzed to compare the number of 2-cells embryos collected at 1.5 dpc and the number of 2-cells embryos obtained after overnight incubation of 1-cell embryos, collected at 0.5 dpc. Genetically modified mouse strains with a similar background (C57BL/6J, B6*129 and FVB/NJ) were analyzed and for strains at a C57BL/6J and B6*129 background, the number of 2-cells embryos obtained after incubation was significantly higher when compared to the number of 2-cells embryos collected at 1.5 dpc (1.4-fold and 1.7-fold, respectively). C57BL/6J wild type mice had similar results with a higher number of 2-cells embryos when collection was performed at 0.5 dpc followed by incubation (1.4-fold). These results can help the planning of 2-cells embryo harvesting by reducing the number of females needed for this procedure.

Keywords

0.5 Dpc Embryos, 1.5 Dpc Embryos, Incubation, Superovulation

1. Introduction

Assisted reproductive techniques are widely used in the laboratory animal field, mainly in mice and rats, as a tool to improve reproductive performance and to reduce animal use. Superovulation is a technique used to increase the number of oocytes that each female ovulates by injecting pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) at an interval of 46 - 48 hours to mimic the effects of FSH and LH, respectively [1]. This technique greatly reduces the number of embryo donor females needed for a given purpose and has been optimized by controlling several factors that can affect its efficiency such as the female's weight [2] [3], age [4], hormone dose [2], interval between hormones administration [2] and estrous cycle phase at the time of hormone administration [5]. For rederivation purposes, superovulation is used to increase the number of oocytes released by each female that can then be collected after natural mating at 0.5 dpc (1-cell embryos) [6], at 1.5 dpc (2-cells embryos) [7] or even at 3.5 dpc (blastocysts) [8] for subsequent embryo transfer. Embryo transfer can then be performed at several stages of embryo development using 1-cell embryo [6] [9], 2-cells embryos [6] [7] [9] or blastocysts [8] [10]. Embryo culture can also be part of the process by collecting embryos at 0.5 dpc and cultivating up to a 2-cells or blastocyst stage.

For cryopreservation, collection of embryos at different phases can also be a possibility after superovulation [11]. Both superovulation and embryo culture have advantages and disadvantages. Although superovulation can improve the efficiency of embryo collection by synchronizing females and reducing the number of animals for a given purpose, it can affect oocyte and embryo's quality. Superovulation has been associated with a negative impact on oocytes and embryos by provoking changes in maternal and paternal imprinted methylation [12] [13] [14] and delaying embryonic and fetal development [14] [15] [16] [17] [18]. It has also been associated with alterations in essential proteins involved in regulation and translation of maternally stored mRNA with short poly A tail, such as poly(A)-binding protein (Epub) and poly(A)-binding protein cytoplasmic 1 (Pabpc1) [16] [19]. Important genes can be dysregulated due to superovulation, such as the Mest gene or the Grb10 [20] [21] and regulatory proteins can also be changed by this procedure like STAT3, leptin, transforming growth factor β -2 among others [22].

Allowing the embryos to grow *in utero* after a superovulation protocol can attenuate the effect of superovulation by decreasing the number of implantation sites [23], reducing the number of living fetuses [16] [18] as well as increasing pre-implantation mortality [14]. PMSG and hCG stimulation causes changes in the uterine environment that negatively affect embryo and fetal development [18] [24], resulting in reduced litter size and foetal organ's weight [25] [26]. These alterations include changes in lipid metabolism [27] and in NK cells [28], whose regulatory effects on implantation can be compromised. Alterations in the placental blood supply and on genes related with glucocorticoid regulation seem to be one of the responsible mechanisms for the decreased pup's weight [25].

Despite the superovulation disadvantages, this is still an important and needed

technique for the collection of large amounts of embryos or oocytes.

Embryo culture causes alterations on gene expression patterns of mouse embryos [29] and on the regulation of transposable elements (TE) and M16 media, frequently used for culture, has been associated with an upregulation of these when compared to KSOM [30].

Using embryo culture before the final application, either cryopreservation or embryo transfer, can have practical advantages such as the fact that only fertilized embryos are transferred into a pseudopregnant female or the possibility of splitting the work session in two or more days.

Literature regarding the best time point for mouse embryo collection is sparse and the timing of embryo collection after superovulation and transfer varies between articles. Van der Auwera [23] shows that when embryos are kept longer inside a superovulated oviduct, the number of implantation sites is lower. However, this was only described in a F1 hybrid CBAxC57BL6 background and no information was available regarding the number of embryos harvested at different time points after superovulation.

Since superovulation and incubation of embryos from different genetic backgrounds are common procedures in rodent animal facilities, and there are no standard protocols regarding the time for embryo collection after superovulation, this study may provide useful information about embryo harvesting.

The herein study aimed to explore the possible reduction on the number of embryos collected after a superovulation protocol during the early phases of development if embryos are kept *in utero* up to a 2-cells stage when compared to collecting the embryos at a 1-cell stage and allowing the passage in the incubator to a 2-cells stage. For that, we analyzed data related to embryo collection from historical data of genetically modified strains at different genetic backgrounds obtained between January 2017 and May 2019 at the i3S Animal Facility and data from C57BL/6J wild type mice.

2. Materials and Methods

2.1. Animals

The experiments were performed at the i3S animal facility and were reviewed and approved by the internal Animal Ethics Committee and by the Portuguese Competent Authority (DGAV) (project reference 2017_03, June 2017). Animals were housed at the i3S animal facility under standard conditions: temperature was kept between 20°C and 24°C and humidity between 45% and 65%; donor females were housed in groups of 3 to 4 females in type II eurostandard filter top cages (370 cm² floor area) for superovulation and then mated with a male, also housed in type II eurostandard cages (males were housed individually during the experimental period); food (2014 Teklad global 14% protein rodent maintenance diets, 2014S, Envigo Spain) and water (type II distilled) were supplied *Ad libitum*; bedding material was corncob and enrichment was also provided with a shelter or card tube and paper for nesting material. Animals were free from the following agents: MHV, EDIM, MPV, MVM, PVM, Sendai virus, TMEV, Ec-

tromelia, LCMV, MAD-1 and 2, Mouse cytomegalovirus, Reovirus, *Bordetella bronquiseptica*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutsheri*, *Cryptosporidium*, *Mycoplasma*, *Pasteurella spp*, *Pseudomonas aeruginosa*, *Salmonella spp*, *Helicobacter spp*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptobacillus moniliformis*, *Klebsiella pneumoniae*, ectoparasites (*Myobia*, *Radfordia* and *Myocoptes*), *Eimeria*, *Entamoeba*, *Giardia*, *Spironucleus muris*, *Tritrichomonas*, *Aspicularis tetraptera* and *Syphacea obvelata* and *muris*. Occasional positives were found at quarantine for *Pasteurella spp*, *Helicobacter spp*, *Klebsiella pneumoniae* and *Staphylococcus aureus* as animal origin differs among the two experiments, as explained on the next section.

Data herein presented arose from the collection of mouse (*Mus musculus*) embryos either for the purpose of embryo transfer (rederivation) or cryopreservation sessions, from January 2017 to May 2019. Data from embryo transfer and cryopreservation sessions are not stated as the aim of this work was to unravel the timing of embryo harvesting. A total of 312 sessions of embryo collection were included in the analysis. Data was grouped according to the genetic background and represent 37 mouse strains of genetically modified mice at a C57BL/6J background, 11 at a mixed background between B6 and SV129 and 6 at an FVB/NJ background housed at the i3S Animal Facility. Another set of experiments was also analyzed using wild type C57BL/6J animals (a total of 2 sessions of embryo harvesting). Animal origin was the i3S animal facility (in case the data correspond to embryo harvesting from cryopreservation sessions) or other facilities in case animals were in quarantine (for rederivation purposes). In that case, in quarantine, animals were mated one week after arrival and the litters from that breeding couple were used for superovulation and mating. For each session of GMM, donor females between 3 and 6 weeks of age from each colony were used. Although superovulation tends to work better in younger females, some females at 6 weeks of age were also used in all groups (harvesting at 0.5 and harvesting at 1.5 dpc) due to the availability of animals and to the fact that females at this age are also described to be used for superovulation [2]. For the C57BL/6J wild type group, females were between 3 and 4 weeks of age. Fertile males (minimum 8 weeks and up to 5 months old) were used. A ratio of 1 female for 1 male was used for mating and males were only used once per week.

2.2. Superovulation

PMSG (PMSG, HOR 272, ProSpec) and hCG (hCG, HOR 250, ProSpec) were diluted at a dose of 50 IU in sterile water and frozen at -20°C until further use. Before injection, aliquots were thawed and diluted in sterile water to get a 100 μL dosage of 5 IU for intraperitoneal injection.

PMSG and hCG were given at an interval of 48h. At the time of hCG injection, females were mated with males overnight and checked for plug in the following morning. Only females with plug were considered for the data analysis to ensure that only oocytes with the possibility of being fertilized were being screened and fertilization was not a determinant factor on the result.

2.3. Embryo Collection

For 1-cell embryo collection, females were euthanized the morning after the mating (0.5 dpc) by cervical dislocation. Oviducts were collected in M2 media [31] and digested in M2 supplemented with 0.5 mg/ml hyaluronidase (Hyaluronidase, H4272, Sigma Aldrich) for less than 1 minute. After digestion, embryos were washed in 50 μ L M2 drops, followed by several washes in 50 μ L KSOM drops (EmbryoMax[®] KSOM Mouse Embryo Media, MR-020P-5F, Sigma Aldrich) and incubated in 50 μ L drops covered with mineral oil (Mineral oil, M8410, Sigma-Aldrich). A maximum of 50 embryos per drop was incubated at 37°C and 5% CO₂. In the following morning, the total number of 2-cells embryos was counted and embryos were then used for cryopreservation or embryo transfer. The initial number of 1-cell embryos was also counted before incubation.

For 2-cells embryo collection, females were euthanized by cervical dislocation at 1.5 dpc. Oviducts were collected in M2 media and flushed with the same media through the infundibulum, using a stereomicroscope (Stemi 2000 C, Zeiss) and a 30G needle. Two-cells embryos were then counted and washed in M2 media before cryopreservation or embryo transfer on the same day. For the wild type (C57BL/6J) session, the number of non-fertilized embryos obtained at 1.5 dpc was also counted.

2.4. Statistical Analysis

Data was analyzed using GraphPad Prism software (version 6.00 for Mac, GraphPad Software, La Jolla California USA). A D'Agostino & Pearson omnibus normality test was used before comparing 2-cells embryos, plug number and fertility rates. For the GMM strains, data were grouped according to the genetic background of each strain and a Mann-Whitney test was performed to compare the number of 2-cells embryos obtained from females with the same genetic background after incubation or after collection at a 2-cells stage. A Mann-Whitney test was also used to compare the mean number of plugs in each background in sessions using incubation and 2-cells embryos harvesting. For the C57BL/6J wild type session, a t-test was used to compare the mean number of 2-cells embryos and fertility rates. Data are shown as Mean \pm SEM and $p < 0.05$ was considered as a statistically significant difference.

3. Results

Cryopreservation and rederivation are techniques that rely on collection of multiple embryos to successfully preserve a mouse strain or transfer to a recipient foster female. We started by collecting the data obtained from rederivation and cryopreservation between January 2017 and May 2019 from the genetic backgrounds most widely used in our animal facility: C57BL/6J, mixed background between C57BL/6J and SV129 (B6*129) and FVB/NJ. The number of sessions per genetic background, the number of genetically modified strains and the number of females used in total are summarized in **Table 1**.

With this data, we aimed at assess whether incubation of 1-cell embryos could

improve the number of 2-cells embryos obtained for each strain. A statistically significant increase of 1.4-fold ($p < 0.0001$) in the number of 2-cells embryos obtained after incubation of 0.5 dpc embryos when comparing to 2-cells embryos collection for C57BL/6J background (**Figure 1(a)**) was found. Similarly, an increase of 1.7-fold ($p < 0.0019$) in the number of 2-cells embryos obtained after incubation for strains at a B6*129 background (**Figure 1(b)**) was observed. For strains with FVB/NJ background, an increase of 1.6-fold in the number of 2-cells embryos was observed after culture when comparing to 2-cells embryos collection (**Figure 1(c)**) although this observation was not statistically significant ($p = 0.0698$). The mean number of plugs between sessions with the same genetic background was not statistically different for strains with a C57BL/6J and B6*129 background ($p = 0.3560$ and 0.5547 respectively). For FVB/NJ background, the mean number of plugs was significantly higher at the incubation group ($p = 0.0438$). Results are summarized in **Table 2** and **Table 3** for the harvesting at 0.5 dpc followed by incubation and 2-cells harvesting groups, respectively.

Table 1. Number of sessions and females analyzed for the different backgrounds from GMM.

	Collection at 0.5 dpc		Collection at 1.5 dpc		Number of GMM strains
	Number of sessions	Number of females	Number of sessions	Number of females	
C57BL/6J	64	299	183	926	37
B6*129	12	60	31	167	11
FVB/NJ	13	97	9	43	6

Number of sessions analyzed for the different backgrounds and number of females used in the total number of sessions according to the method of 2-cells harvesting: collection at 0.5 dpc followed by incubation and collection at 1.5 dpc.

Table 2. Data from GMM for 0.5 dpc followed by incubation groups at different backgrounds.

Background	Mean number of plugs per session \pm SEM	Mean number of 1-cell embryos \pm SEM	Mean number of 2-cells embryos \pm SEM	Mean fertilization rates (%) \pm SEM
C57BL/6J	4.672 \pm 0.2826	22.72 \pm 1.383	13.23 \pm 0.9228	59.96 \pm 2.331
B6*129	5.000 \pm 0.5222	25.16 \pm 3.351	16.86 \pm 2.716	66.76 \pm 5.689
FVB/NJ	7.462 \pm 0.8444	19.88 \pm 3.853	10.02 \pm 1.867	58.88 \pm 8.099

The mean number of 1 and 2-cells embryos per session is based on the number of embryos obtained divided by the number of females used per session.

Table 3. Data from GMM for 1.5 dpc harvesting groups at different backgrounds.

Background	Mean number of plugs \pm SEM	Mean number of 2-cells embryos \pm SEM
C57BL/6J	5.070 \pm 0.2350	9.3 \pm 0.7463
B6*129	5.3870 \pm 0.3953	9.078 \pm 1.731
FVB/NJ	4.778 \pm 0.8625	6.377 \pm 1.666

The mean number of 1 and 2-cells embryos per session is based on the number of embryos obtained divided by the number of females used per session.

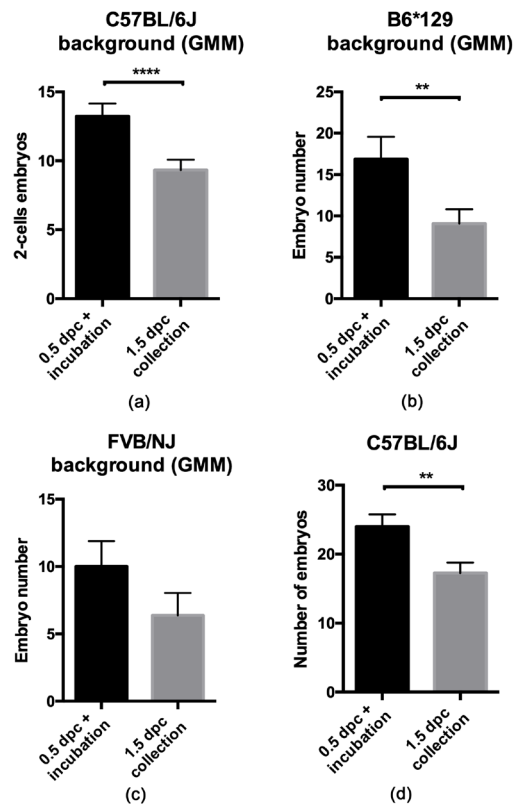


Figure 1. Mean number of 2-cells embryos obtained from collection at 0.5 dpc followed by incubation and 1.5 dpc collection at different backgrounds. Mean number of 2-cells embryos obtained from GMM or wild type strains using collection at 0.5 dpc followed by incubation and 1.5 dpc collection for: (a) C57BL/6J background (GMM); (b) B6*129 background (GMM); (c) FVB/NJ (GMM) background; and (d) C57BL/6J wild type. Results are expressed as Mean \pm SEM.

When analyzing data obtained through the collection of 0.5 dpc followed by incubation and harvesting at 1.5 dpc from wild type animals (C57BL/6J), the number of 1 and 2-cells embryos was counted for both groups. A significant difference ($p = 0.0093$) was found between the mean number of 2-cells embryos collected at 1.5 dpc and at 0.5 dpc followed by incubation. The mean number of 2-cells embryos had an increase of 1.4-fold when incubation of 0.5 dpc embryos is used. No significant difference was found between the two groups fertilization rates ($p = 0.1898$). Data are summarized in **Table 4** and **Figure 1(d)**.

The results herein presented suggest that, to obtain 100 fertilized embryos, we can reduce the number of superovulated females for the majority of the GMM strains analyzed, if the embryos are collected at 0.5 dpc and culture to 2-cells stage. For C57BL/6J, B6*129 and FVB/NJ strains are required 40%, 47% and 46% less females, respectively (**Table 5**). For C57BL/6J wild type animals, similar results are found. In this strain, incubation after 0.5 dpc collection leads to about 33% less females (**Table 5**).

Together, these results reinforce that, at least for the backgrounds analyzed, incubation of 1-cell stage embryos can increase the number of 2-cells stage embryos, thus, reducing the number of superovulated females.

Table 4. Data from C57BL/6J for 0.5 dpc followed by incubation and 1.5 dpc harvesting groups.

Collection at 0.5 dpc				Collection at 1.5 dpc			
Mean number of 1-cell embryos \pm SEM	Mean number of 2-cells embryos \pm SEM	Fertility rate (%) \pm SEM	Number of females	Mean number of non-fertilized embryos \pm SEM	Mean number of 2-cells embryos \pm SEM	Fertility rate (%) \pm SEM	Number of females
41.50 \pm 3.122	24.00 \pm 1.770	59.88 \pm 3.742	22	8.813 \pm 1.285	17.25 \pm 1.529	67.09 \pm 3.674	16

The mean number of 1 and 2-cells embryos was obtained individually, per female.

Table 5. Number of females needed for the collection of 100 embryos using the two harvesting methods for GMM at different backgrounds and wild type mice from the C57BL/6J strain.

		Collection at 0.5 dpc	Collection at 1.5 dpc
GMM	C57BL/6J	7	12
	B6*129	6	12
	FVB/NJ	11	20
Wild type	C57BL/6J	4	6

4. Discussion

Superovulation is a technique used to increase the number of oocytes produced and released by female mice. It has the advantage of decreasing the number of females needed for the collection of oocytes or embryos, contributing to the application of one of the 3R's. Despite the potential of this technique, superovulation has many times an uncertain outcome because the number of oocytes released by the female can be low [2] [4] [5] and also the quality of the oocytes or embryos produced after the injection of high doses of PMSG and hCG can be reduced [12] [13] [14] [16] [17] [18] [21] [22] [24] [27] [32] [33]. Our results show that, at least in strains with a C57BL/6J, B6*129 or with an FVB/NJ genetic background (although this last one was not statistically significant), it is more effective to collect embryos at a 1-cell stage (0.5 dpc) and culture to a 2-cells stage when compared to collecting embryos at a 2-cells stage (1.5 dpc) after female's superovulation. Similar results were achieved by Van der Auwera *et al.* [23] that considered that the oviductal *milieu* after superovulation have a negative impact on embryo development when compared to a non-stimulated oviduct or to *in vitro* culture using B6XCBA donors and NMRI pseudopregnant females. Collecting the embryos at the 1-cell stage can significantly increase the number of embryos obtained and thus, reduce the number of animals required for this type of protocols. The effect observed on C57BL/6J, B6*129 and FVB/NJ can be related to the fact that, after superovulation, embryo development is compromised and it is possible that removal of embryos from the uterine environment decreases the negative effect of superovulation by decreasing the exposition to PMSG and hCG. Taken into account that PMSG has an half-life of 40 to 120 h and the half-life of hCG is about 24 - 36 hours [27], a possible explanation for our results are the lower levels of PMSG and hCG exposition once embryos

are removed from the uterine environment and incubated in KSOM after several washes in M2. GMM strains with an FVB/NJ background didn't produce a statistically significant difference but this can be related to the smaller sample size available for this strain. Using a wild type strain such as the C57BL/6J, the same tendency of reduction in the number of 2-cells embryos when 1.5 dpc harvesting is used was noticed. The number of 2-cells embryos collected after incubation or even at 1.5 dpc is higher when compared to the same method using data from GMM strains at a B6 background. This can be explained by the fact that females used in the C57BL/6J experiment were all at an optimal age (3 - 4 weeks), resulting in higher number of oocytes produced after superovulation and also because this experiment resulted from only one wild type strain, having so, less variability. Nevertheless, thorough investigation will be needed to clarify the reasons why the number of 2-cells embryos is higher when incubation is used and to identify the changes observed on gene expression, lipid metabolism or the role of other essential mechanisms for embryo growth which are changed when superovulation is used and comparing it at a 1-cell and at a 2-cells stage. It would also be interesting to compare these results with embryos collected from females that are naturally mated, without superovulation. Our results are, however, compatible with those described by Van der Auwera *et al.* [23], reinforcing that the superovulation impact occurs not only at a later stage of development but also that the embryo reduction occurs soon after superovulation.

Genetic alterations among the strains used may also contribute to variable responses to superovulation, fertilization levels [34] [35] and incubation rates. Fertilization rates can be easily determined when embryos are collected at 0.5 dpc by considering the total number of oocytes/1-cell embryos and allowing passage to a 2-cells stage or detecting pronucleus under the microscope. However, when the harvesting is performed at 1.5 dpc, the total number of embryos counted (including non-fertilized and fertilized embryos) is lower, as shown in **Table 4**, suggesting that part of the embryos might be reabsorbed during the night. For that reason, the number of 1-cell embryos/oocytes obtained at 0.5 dpc and the number of non-fertilized embryos collected from the C57BL/6J wild type group were not compared but fertilization rates were not significantly different ($p = 0.1898$). This allow us to conclude that the effect observed on the reduction of the number of 2-cells when harvesting is performed at 1.5 dpc is not related to lower fertilization rates. For the GMM strains, the number of plugs was not different among groups and the fertilization rates for the 0.5 dpc groups was similar to the fertilization rates obtained with the wild type strains. Further data would also be needed to determine if this effect also occurs in other commonly used mouse strains.

5. Conclusion

Mouse superovulation can be optimized by controlling factors such as the female age, weight, dose and time of hormone administration. Our results show, that at least for the strains studied, a better planning of experiments involving the use of

2-cells embryos through incubation technique can significantly decrease the number of females needed to obtain a specific number of embryos. Thus, when no specific stage of embryo development is required (such as for embryo transfer for rederivation or cryopreservation), harvesting embryos at 0.5 dpc may provide a better outcome in the number of embryos than collecting embryos at 1.5 dpc (2-cells stage embryos). This practice may display a very positive impact on the reduction of animals used for superovulation contributing actively to the 3R's goals.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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