Theriogenology 84 (2015) 1306–1313

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

The role of diet and housing-temperature in the production of genetically modified mouse embryos and their developmental capacity after cryopreservation

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A R T I C L E I N F O

Article history:
Received 30 March 2015
Received in revised form 16 June 2015
Accepted 8 July 2015

Keywords:
Cryopreservation
Embryo production
Embryo transfer
Phytoestrogen
Sex ratio
Temperature

A B S T R A C T

Mutant mouse lines are unique models with an enormous scientific potential. Cryopreservation of preimplantation embryos or of spermatozoa is a common approach to save those lines. The breeding of a line can be discontinued if sufficient specimens have been cryopreserved. Prerequisites to economically cryopreserve embryos are high yields of embryos prepared from donors and a high recovery rate after revitalization. Diets for laboratory animals are often produced from phytoestrogen-containing soy; the present study shows that feeding the donor animals with a phytoestrogen-poor diet is more efficient compared to a phytoestrogen-containing, soy-based diet. Additionally, a uterotrophic bioassay indicating the estrogenic role of compounds showed a significant increase of the relative uterus size of females fed with a phytoestrogen-rich diet. The role of the housing-temperature was investigated, too, showing that a housing-temperature of 24 °C results in the best embryo yields. The production of two-cell embryos is more economic than the production of eight-cell embryos. Investigating the recovery rate of frozen/thawed embryos, a very high recovery rate was determined when both, two- and eight-cell embryos were thawed. However, the capacity to develop to the next embryonic stage *in vitro* was dramatically reduced when two-cell embryos were compared to eight-cell embryos. After embryo transfer, the sex ratio became uneven and more males were delivered. This effect might be due to the procedures to which animals and embryos were subjected. These data show that many parameters can influence the production of animals when using (frozen/thawed) embryos. These parameters need continuous surveillance.

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1. Introduction

In recent years, genetically modified (GM) mouse lines have become one of the most important animal species. Producing and characterizing GM lines involves tremendous effort, the number of lines is increasing rapidly, and their scientific value is enormous. Several limitations have to be considered when working with these animals: small colony sizes, the continued danger of loss, a limited availability and breeding success, the need to keep these lines in stock, and complex import procedures. Cryopreservation of preimplantation embryos is a common approach to save GM lines while avoiding the need of a breeding nucleus.

Cryopreservation and embryo transfer are established methods, consuming many embryo donors but leading to also a microbiologically safe recovery. Therefore, the yield of embryos should be optimized; negative influences must be determined and eliminated. Improvement studies should include the recovery rate of frozen/thawed material.

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0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.07.010

We have already published several studies on those factors, such as the role of the genetic background, the age of males, or the facility [1]; of pheromones, mating frequency, hygiene conditions, or annual rhythms [2]; or of environmental factors such as humidity or noise [3]. To better understand other embryo yield–influencing parameters, diet compounds might be of interest. Many diets for rodents are based on soy, alfalfa, and other phytoestrogen-containing plants, possibly resulting in an increase of the phytoestrogen content (e.g., genistein, daidzein). They interact like estrogens with the corresponding receptors. Importantly, phytoestrogens are described to influence the fertility and puberty in female and male mice [4–9] which might have an impact on the production of embryos, especially when superovulating female donors [10,11]. A possible estrogenic effect can be determined by calculating the relative uterus weight using a uterotrophic bioassay [12–14].

Another environmental influence might be the housing-temperature. Animal facilities must operate at a stable temperature; changes are uncomfortable for both animals and staff and are influencing their homeostasis. Handbooks recommend a standard housing-temperature of 20 °C to 24 °C for mice [15], in practice very often 22 °C. A slightly higher temperature might trigger the embryo production [16]. The role of moderate temperature changes remains to be elucidated.

Superovulation of donors leads to a higher rate of early preimplantation embryos; the number of embryos with a capacity to develop is reduced *in situ* from stage to stage. Subsequently, the preparation of earlier embryonic stages leads to a higher embryo yield. Because the aim of cryopreservation is to archive and recover a line efficiently, the developmental capacity of frozen/thawed embryos cryopreserved in different stages of development remains to be investigated, i.e., if limiting factors as e.g., the two-cell arrest [17,18] might better be overcome before cryopreservation or after revitalization.

The sex ratio is a major parameter in population genetics. In humans, the number of newborn males is slightly higher (worldwide 1.05 male compared to 1.0 female [19,20]). In that matter, only a few studies on mice were published not showing significant differences [21,22].

In the current report, we studied the developmental capacity of preimplantation embryos by investigating the housing-temperature and the diet on the embryo yield, the revitalization success, the capacity to develop *in vitro*, and the sex ratio after embryo transfer.

2. Materials and methods

2.1. Animal experimentation

All mice used in this study were housed in the animal facility of the German Cancer Research Center (DKFZ), Heidelberg, Germany, according to standard procedures described elsewhere [23]. Genetically modified mouse lines originally received from different sources were bred and expanded in-house, whereas wild-type (WT) mice with corresponding genetic backgrounds (C57BL/6; NMRI) were received from Charles River (Sulzfeld, Germany). Individually ventilated caging systems and barrier facilities (with open caging systems) were used as described in detail previously [2,3]. In the temperature experiment, ventilated cabinets with a controlled housing-temperature were used. Scantainer 48-VS-III was used for housing at 24 °C and 26 °C, Scantainer B110 for housing at the standard temperature of 22 °C (Scanbur, Karlslunde, Denmark).

The age of the male mice used ranged from three to nine months. Males were housed singly, and females were kept in groups of five. The health of the animals was monitored according to the Federation of European Laboratory Animal Science Associations recommendations [24]. If not mentioned otherwise, the animals were fed with a phytoestrogen-poor diet *ad libitum* (Table 1). Animal experimentation was performed according to the German Animal Welfare Act. All animal experiments were approved by the Animal Welfare Department of the Competent Authority (Regierungspräsidium Karlsruhe, Germany) and conducted under the surveillance of the intramural Animal Welfare Committee of DKFZ.

2.2. Diet

All animals (males and females) were fed with a phytoestrogen-poor or phytoestrogen-rich diet *ad libitum*. The phytoestrogen-poor diet was provided from the time point of weaning or import. Diets used before were not documented in all cases. Details of all diets used are shown in Table 1; they were produced by Altromin (Lage, Germany), Kliba (Kaiseraugst, Switzerland), and LASvendi (Soest, Germany). Analyses of the contents of the diet batches used are shown in Table 1; these data were provided by the manufacturers. Because of legal reasons, these data remain anonymized.

2.3. Production of embryos

Three- to five-week-old donor females on C57BL/6 background were used and superovulated (7-IU PMSG at 4 PM; two days later, 7-IU hCG at 12 PM). Hormones were prepared weekly and dissolved in PBS, portioned in 1 mL samples, and stored at −20 °C. These samples were thawed directly before use, and any remains were discarded. Vaginal plug–positive (VP^+) females were separated at Day

Table 1
Details of all diets and analyses of the contents of the diet batches used in the study.

Diet	$phyto^+$	$phyto^-$ (1)	$phyto^-$ (2)	$phyto^-$ (3)
TGE (mg/kg)	172.7	55.5	n.s.	<20
Total genistein (mg/kg)	110.0	29.5	40.0	n.s.
Total daidzein (mg/kg)	65.8	30.8	12.0	n.s.
GE (MJ/kg)	16.1	16.4	n.s.	15.9
ME (MJ/kg)	13.5	13.4	11.2	13.1
Crude fat (%)	4.5	5.3	4.0	4.3
Crude protein (%)	18.5	18.2	19.1	16.9

Diets used: "$phyto^+$" is phytoestrogen rich, "$phyto^-$" is phytoestrogen poor. (1), (2), and (3) indicate the diets of different origins.
All data show analyses of the diet batches used according to the manufacturers' information.
Because of legal reasons, the origins of the diets are anonymized.
Abbreviations: GE, gross energy; ME, metabolizable energy; n.s., not specified; TGE, total genistein equivalent.

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