Lipid droplet utilization by the mouse embryo

S Bisogno[1], R Arena[1], K Fic[1], Ł Gąsior[1] and GE Ptak[1,2]

[1]Malopolska Centre of Biotechnology, Jagiellonian University, Krakow Poland
[2]Faculty of Biosciences, University of Teramo, Italy

Corresponding author email: g.ptak@uj.edu.pl

Abstract

High numbers of lipid droplets (LDs) in mammalian eggs are stored and maintained throughout embryo development without marked signs of their utilization. It was previously demonstrated in large domestic mammals that removing lipids from the zygote does not influence post-implantation development in terms of the rate of delivered offspring. Previously studied pig and cow eggs contain considerable amounts of LDs, while mice have a very low level of ooplasmic lipids, which allows to more precisely analyze any effect of lipid removal on developmental dynamics *in vitro*. We wanted to know if lipid fraction removal would influence the dynamics of preimplantation development of mouse embryos. To do this, mouse zygotes were mechanically delipidated and their progression to the blastocyst stage was evaluated *in vitro*. Part of blastocysts were transferred to pseudopregnant females for development to term, and then offspring health parameters were evaluated. Our experiments showed no effects of lipid removal on the rate and timing of mouse embryo development. Furthermore, there were no differences in post-natal characteristics of offspring developed from delipidated and non-delipidated embryos. In conclusion, normal developmental progression of dilapidated embryos indicates that LDs are largely unutilized during preimplantation stages. The apparent dispensability of the LDs fraction throughout preimplantation development prompts questions about their so far uncovered role in mammalian embryo. Based on our preliminary observation of the ultrastructure of diapaused blastocysts from mouse and sheep, we speculate that LDs are utilized by the embryo during delayed implantation, i.e., while waiting for the maternal receptivity signal before implantation.

Keywords: embryonic diapause, lipid droplets, blastocyst, mouse

Introduction

Mammalian embryos contain considerable numbers of lipid droplets (LDs), which are intracellular sites for the storage of triglycerides and steryl esters. Generally, in mammalian cells, LDs are critical for cellular metabolism, the generation of signaling molecules and energy homeostasis. However, in early mammalian embryos, ATP requirements are met by the utilisation of carbohydrates [1]. Without carbohydrates, mammalian embryos arrest their development quickly: within 1-2 cleavage divisions in mice [2]. On the other hand, there are no major needs for the energy stored in LDs, as after each cleavage division, blastomeres become smaller and smaller and the growth of the mammalian embryo starts only at the blastocyst stage. Once the blastocyst hatches, the embryos

naturally slow down their growth [2], and in the absence of uterine crosstalk, they will not develop further. Maternal recognition of the embryo is followed by implantation, when maternally provided energy resources start to be used.

It was suggested that LDs stored in the mammalian egg are dispensable for embryo development, as their removal from one-cell stage pig and bovine embryos does not influence their further development [3,4]. In both previous studies, delipidated embryos were directly transferred to surrogate mothers for development to term, and no differences in the rate of offspring obtained from delipidated and control embryos were found. Relevantly, preimplantation embryo development was not evaluated. Both previous studies utilized models (pig and bovine eggs), in which lipid fractions represent a substantially higher amount than in mouse egg. These issues prompted us to verify if the removal of lipids does indeed influence preimplantation embryo development in mammalian eggs, using a species with one of the lowest observed quantities of lipids, the mouse.

Material and Methods

Animal studies. All experimental procedures were conducted according to the guidelines of European Community regulation 86/609 and conformed to the Polish Governmental Act for Animal Care. Animal procedures were conducted at the Malopolska Center of Biotechnology and the Institute of Zoology and Biomedical Research of Jagiellonian University (Kraków), permission number 123/2018 approved by II Local Ethical Commission in Kraków. Mouse experiments were performed on C57BL/6xCBA mice; 3 to 6 month-old females and males were used. Animals were maintained in a temperature- and light-controlled room (22°C; 12 h light-dark cycle) and were provided with food and water *ad libitum*.

Embryo collection, manipulation and culture. Female mice were superovulated, PMSG (5IU) was injected, hCG (5IU) was applied 48 hours later and the femaled were mated 1:2 to male mice of proven fertility and the same strain. At 23 hours after hCG injection, all female donors were euthanized by cervical dislocation and oviducts were collected and placed in a dish with 0.5 ml of M2 medium; the ampulla was torn open to release the zygotes. The zygotes were centrifuged at 7000 x *g* for 12 minutes at 37°C in 500 µl of M2 medium supplemented with 2.5 µl of cytochalasin B. After centrifugation, the zygotes were washed three times in M2 medium and the zygotes were placed in KSOM medium for 1 hour recovery. Then, the zygotes were placed in KSOM supplemented with cytochalasin B. The zona pellucida was cut and then the fraction of lipids (Delipidation, AD) or the part of cytoplasm (Control manipulation, CTR) was removed. Removal of lipids was carried out using an aspiration pipette (diameter 12-15 µm) attached to the manipulator (MMO-4, Narishige, Tokyo, Japan) under an inverted microscope (DMi8, Leica). After manipulation, the zygotes were washed three times in KSOM and incubated at 37°C 7% O_2, 5% CO_2 for 4 days in a drop of 20µl KSOM under mineral oil (Sigma-Aldrich) that had been equilibrated to 37 °C at 7% O_2 and 5% CO_2 overnight.

LD staining by BODIPY 493/503 and Nile Red. The blastocysts were fixed in 4% Paraformaldehyde for 20 minutes and then washed 3 times in 0.4% PBS-PVB. For LD staining, the fixed blastocysts were incubated with 1 µg/ml BODIPY 493/503 (Invitrogen™) and 10 µg/ml Nile Red in 0.4% PBS-PVP for 1 hour, and then washed three times in 0.4% PBS-PVP. Mounted specimens were analyzed with a ZEISS LSM 880 Confocal Laser Scanning Microscope, using a 20x Zeiss Plan-Apochromat Infinitive corrected objective with a numerical aperture of 0.8. Relative fluorescence signal intensities were analyzed using ImageJ and normalized to control at 4.5 days post coitum (dpc).

Embryo transfers and offspring evaluation. Zygotes obtained after natural mating of animals were cultured *in vitro* until the morula-stage (3.5 dpc). Embryos were transferred to pseudopregnant recipient females at 3.5 dpc. Recipients were mated with vasectomised males a day after donor

mating. Recipients with vaginal plugs were selected for embryo transfer and anesthetized. After cleaning and shaving the back of the animal, a small incision was made and the uterus was exposed. A small hole made with the needle in the uterine wall was used to allow transfer of embryos with a glass pipette. The embryo transfer procedure was performed into both horns of the uterus, 5 embryos for each horn. Finally, the body cavity was sutured and the female transferred to a warm and clean cage. Females were allowed to deliver naturally. Physical landmarks were evaluated in offspring: incisor eruption and fur appearance at post-natal day (PND) 7, eye/ear opening at PND 14, body weight was recorded at PND 2 and 7 and then, every 7 days until PND 28.

Transmission electron microscopy (TEM). Diapaused blastocysts from mouse and sheep obtained during a previous unrelated study [8], were fixed in 2.5% glutaraldehyde overnight at 4°C, washed in 0.1 M cacodylate buffer, and treated in 1% OsO_4 for 1 h. Samples were dehydrated through a graded series of ethanol, then infiltrated in pure resin (Poly/Bed 812, Polysciences, Inc.) through increasing concentrations of resin in 100% propylene oxide, propylene oxide: resin 3:1 for 1 h and 1:1 overnight at room temperature. The samples were transferred into pure resin, and polymerization was carried out at 60°C for three days. Sections of ~70 nm were cut with an ultramicrotome (Leica) and contrasted with uranyl acetate and lead citrate. Images were captured with the Tecnai Osiris 200kV (FEI). LDs area was quantified using the ImageJ segmentation tool (NIH).

Statistical Analysis. The rate of embryo development was compared using the Fisher's exact test. The mean of the fluorescence intensity was compared using a *t*-test. All means are presented ± standard error of the mean (S.E.M.). Differences with probabilities of $p<0.05$ were considered significant.

Results and Discussion

The rate and timing of embryo development, in terms of reaching progressive stages of preimplantation development was similar among groups (Fig. 1C). The percentage of 2-cell stage embryos at 1.5 dpc from delipidated group, AD (98,5%, 64/65) was comparable to that from CTR (94,1%, 48/51), as well as the percentage of 4-8 cell stage embryos in AD (72,3%, 47/65) and CTR group (72,5%, 37/51). There were no differences in the rate of morulae: AD (70,8 % 46/65) vs. CTR (60,8%, 37/51), and blastocyst rate of AD group (64,6 %, 42/65) vs. CTR (60,8%, 31/51). Our finding demonstrated no effect of LDs removal on the developmental dynamics of delipidated mouse embryos. As demonstrated by the fluorescence staining of LDs using two different dyes, BODIPY and Nile Red, we were able to mechanically remove substantial parts of LDs, however not the entire fraction (Fig. 1D). This indicates that after centrifugation not all of LDs were concentrated in order to form a characteristic button located on the surface of the cytoplasm (Fig. 1B). Offspring obtained following delipidation of zygotes showed normal body weight during pre-weaning period and no differences in physical landmarks such as incisor eruption, fur appearance and ear/eyes opening were recorded (Fig. 2B). Although the number of evaluated offspring was limited in this study (as it was out of the scope of the project), our data in mouse further confirm previous findings in the pig [3] and cow [4], that the delipidation does not influence postimplantational development of embryos. Altogether, the removal of lipids from mouse zygotes did not affect the timing or proportion of embryos and offspring during preimplantation, as well as postimplantation development.

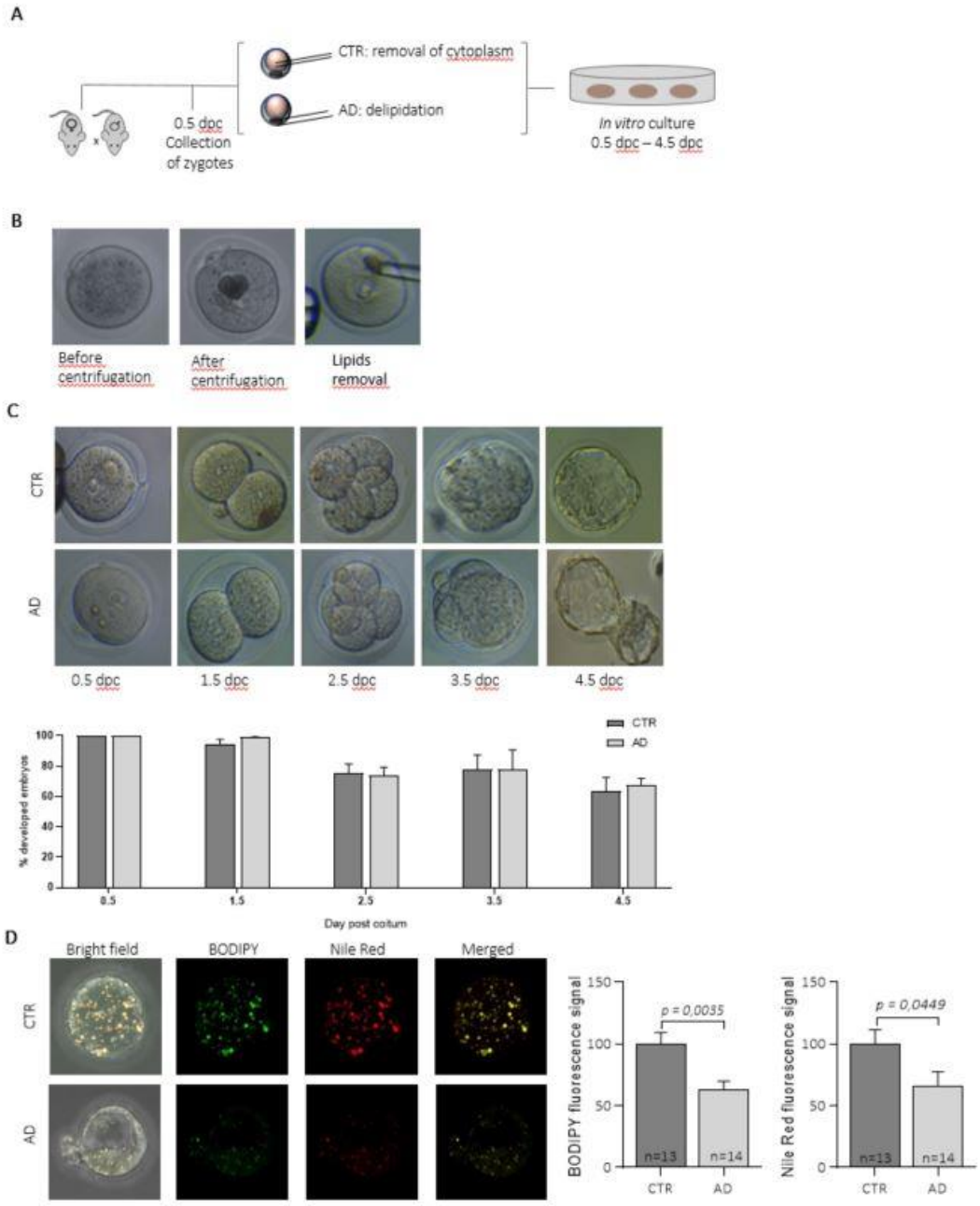
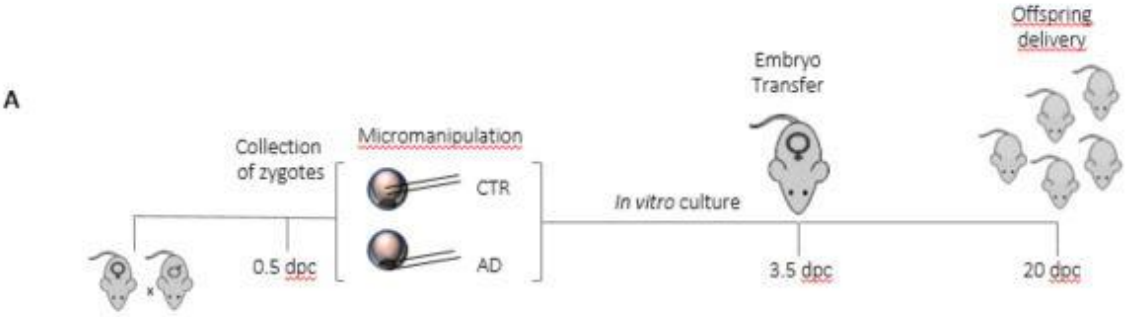
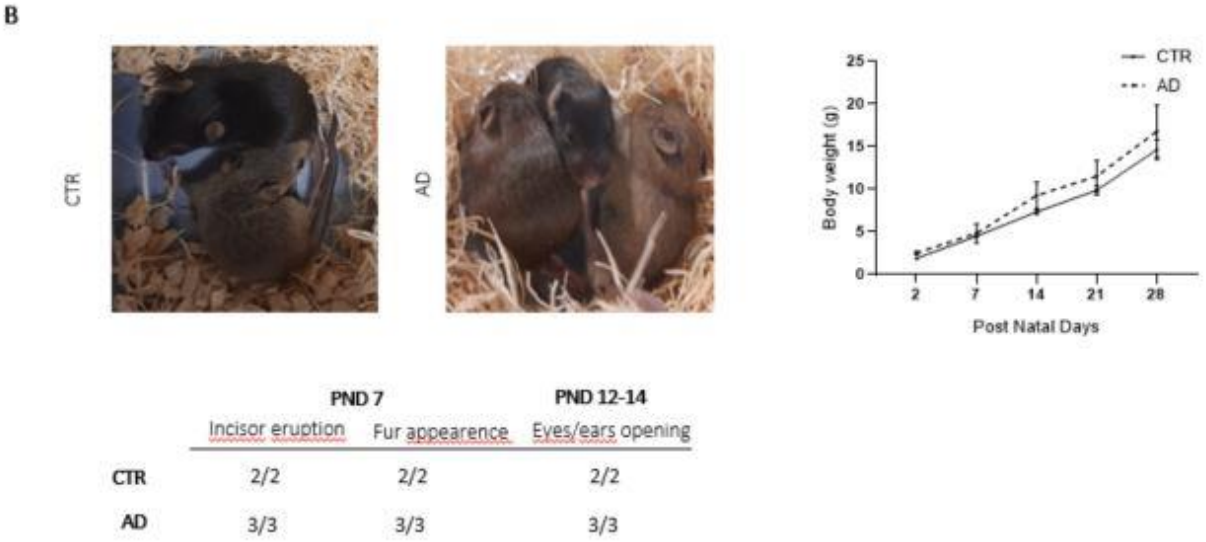


Figure 1. Temporal dynamics of preimplantation development of delipidated mouse embryos A. Experimental Plan: animals were mated and, at 0.5 days post coitum (dpc), the zygotes were collected, centrifuged and manipulated in order to remove lipids fraction (after delipidation, AD group) or the part of cytoplasm (control, CTR). Embryos were than cultured for 4 days in KSOM at reduced oxygen atmosphere (7%). **B.** The panel shows representative images of a zygote, from the left: after collection, after centrifugation and during the removal of lipids. **C.** Developmental progression of embryos obtained by LDs removal and controls: upper panel shows a representative images (20x magnification) of embryos developed to each evaluated stage; lower graph shows the proportion of developed embryos for each stage. Data show non-significant differences among the groups for each stage (Fisher's exact test). Data shown in the graph are mean ± SEM. **D.** Lipid content in the blastocysts developed from zygotes after delipidation (AT) or partial removal of cytoplasm (CTR). Representative images (20x magnification) of the blastocysts in bright field, stained with BODIBY 493/50 and Nile Red, and merged. Graphs show the mean of the fluorescence intensity measured as a mean grey value obtained from the blastocysts (ImageJ). Data are presented as the mean ± SEM. Values show mean grey value normalized to the mean intensity observed in CTR group (arbitrary units). Values differ significantly ($p<0.05$), t-test.





	PND 7		PND 12-14
	Incisor eruption	Fur appearence	Eyes/ears opening
CTR	2/2	2/2	2/2
AD	3/3	3/3	3/3

Figure 2. Post-natal development of delipidated embryos. A. Work plan: Zygotes were collected from the oviducts of previously mated females, centrifuged and manipulated in order to remove lipids fraction (after delipidation, AD group) or the part of cytoplasm (control, CTR). Manipulated embryos were than cultured *in vitro* for 3 days and transferred to pseudopregnant females for development to term of pregnancy. **B.** Representative pictures of obtained offspring. The table below shows the physical landmarks of offspring: incisor eruption, and fur appearance at 7 day of age and ears/eyes opening at second week of age. No differences among the groups were recorded. The graph shows the comparison of body weight in pre-weaning period between CTR and AD group. Data are presented as the mean ± SEM. No significant differences after two-way ANOVA.

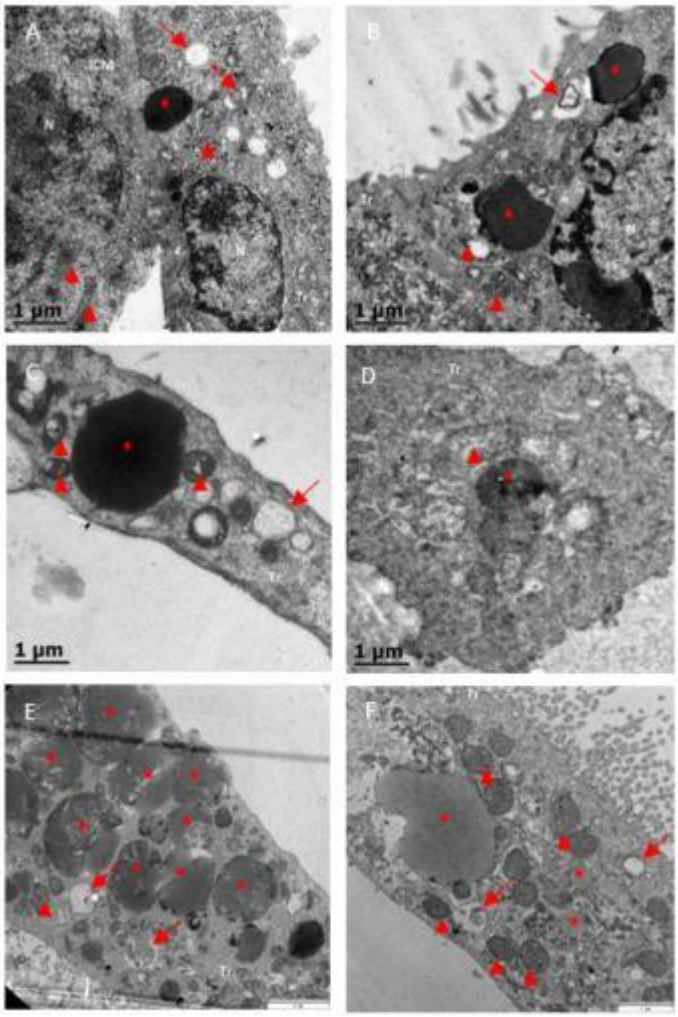


Figure 3. Transmission electron microscopy images of control and diapausing blastocysts from mouse and sheep. A. control blastocyst collected from pregnant mouse at 4 dpc; **B.** diapausing blastocyst collected from ovariectomised mouse at 6 dpc; **C.** diapausing blastocyst collected from ovariectomised mouse at 8 dpc; **D.** diapausing blastocyst collected from ovariectomised mouse at 10 dpc; **E.** control blastocyst collected from sheep at 7 dpc; **F.** diapausing blastocyst collected from ovariectomised sheep at 15 dpc. TEM show lipid droplets in close proximity to autophagic vacuoles and to mitochondria or closely attached to them in diapaused blastocysts in both species, mouse and sheep (red * indicate lipid droplets, arrowhead mitochondria, arrow autophagic vacuoles, N nucleus, Tr Trophoblast, ICM inner cell mass).

Our findings support previous suggestions of the dispensability of cytoplasmic lipids during preimplantation embryo development. Coherent anti-Stokes Raman scattering microscopy observation of LDs during mouse development up to the blastocyst stage also showed that lipid content remains largely unchanged at preimplantation stages [5,6]. While dispensable for preimplantation embryo development, we speculate that cytoplasmic LDs play a crucial role at the blastocyst stage, when the embryo does not implant directly into the maternal uterus, but instead has to wait due to a lack of maternal receptivity signal, such as during delayed implantation. We speculate that delayed implantation is possible because energy reserves are accumulated in blastocyst in the form of LDs. Our preliminary analysis of transmission electron microscopy images of blastocysts collected from mice and sheep during experimentally induced delay of implantation (by bilateral ovariectomy [8]) showed that diapausing blastocysts remain metabolically active (Fig. 3).

Ultrastructural features related to the activation of lipid metabolism in diapausing blastocysts, are reflected by size reduction of LDs in sheep and close contact acquisition between mitochondria and LDs and autophagosomes in diapausing mouse blastocysts. Autophagy, and specifically lipophagy (selective autophagy of LDs) in diapausing embryos is evidenced by the presence of autophagosomes having LDs as their selective cargo. A high level of autophagy was previously reported in diapausing mouse embryos [7]. Altogether, the ultrastructural features of diapausing blastocysts indicate the active utilization of LDs during delayed implantation. This preliminary finding, if confirmed, would indicate a so far uncovered role of LDs in mammalian eggs. Our hypothesis, if confirmed, would have crucial implications for the understanding of embryonic diapause. Embryonic diapause may be induced in so-called non-diapausing species [8,9]. In this view, intracellular conservation of adequate lipid content would be relevant to any mammalian species. It would shed new light on the process of ED in mammals, and clarify the so far unknown role of intracellular lipids during preimplantation embryo development.

Declaration of interests

The authors declare no competing interests.

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Author Contributions

SB performed embryo micromanipulations and culture, RA performed TEM analysis, KF performed embryo transfers and animal experiments. ŁG performed confocal analysis and helped with embryo micromanipulations, GEP conceived the project, designed the experiments, and wrote the manuscript with feedback from all authors.

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