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Liposomal Particle Kinetics in Mouse Amniotic Fluid

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Drug delivery systems, drugs are administered into the amniotic fluid, may contribute to fetal therapy. The present study explored changes in the volume of amniotic fluid from E11.5 through E17.5, and kinetics of calcein-containing liposomal particles incubated in the amniotic fluid collected at E12.5, E14.5, and E16.5 in Jcl/ICR mice. The volume of amniotic fluid increased until E15.5, followed by a sharp decrease at E17.5. The calcein release rate remained within 4% until day 14 in serum and each amniotic fluid. No significant differences were noted in the release rate between the serum and amniotic fluid at various time points. Therefore, the calcein release rate of liposomal nanoparticles in the amniotic fluid remained low and did not differ from that in the serum for as long as 14 days at different time points during gestation. These observations suggest that liposomal nanoparticles remain stable for prolonged periods in the amniotic fluid.

Keywords: liposomal particle, amniotic fluid, mouse

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INTRODUCTION

Recent advances in medical science have expanded the range of available prenatal treatment options. Through prenatal treatments, such as protein and enzyme replacement, for inherited metabolic birth defects and respiratory distress syndromes due to premature birth, diseases may be treated and fetal organ development may be promoted by administrating drugs, proteins, and enzymes in the amniotic fluid.

Since their discovery by Bangham AD in 1964 [1], liposomal delivery systems have been used to treat infectious diseases and cancer due to their low toxicity, high biocompatibility, and controllable release kinetics [2–4]. In the subsequently developed method of coating surfaces with polyethylene glycol (PEG), slow clearance of liposomes allows for their stabilization [5,6], which contributed to chemotherapy. The properties of these liposomal particles may be suitable for fetal therapy, wherein drugs and other substances are administered into the amniotic fluid, the quantity and quality of which changes during pregnancy [7]. Direct administration of drugs into the amniotic fluid may realize therapeutic effects by reaching fetal organs. However, little is known regarding the kinetics of liposomal particles in the amniotic fluid.

Here, we observed changes in the volume of amniotic fluid and kinetics of liposomal particles in the amniotic fluid of mice, as their placenta is hematogenous and similar in structure to the human placenta [8].

MATERIALS AND METHODS

Animal experiments

Jcl/ICR mice (CLEA Japan, Tokyo, Japan) between 8 and 20 weeks of age were used. The mice were housed at the Department of Experimental Animals, Center for Integrated Research in Science, Shimane University. The animals were maintained under controlled conditions with constant temperature and humidity and a 12-12 h light-dark cycle. One male and one female were mated overnight in the same cage. The noon of the day when a vaginal plug was observed was defined as embryonic day (E) 0.5.

Pregnant mice from E11.5 through E17.5 were sacrificed under deep anesthesia using Medetomidine, Midazolam and Butorphanol, and the weight of the embryo and placenta and volume of the amniotic fluid were measured (n = 6). Due to its small amount, the amniotic fluid was measured by weight, not volume. Specifically, the fetus, wrapped in amniotic membrane, was removed from the placenta, and the amniotic membrane was torn open on the foil. The amniotic fluid was collected in the foil and its weight was measured. Three independent litters were analyzed. The amniotic fluid for the evaluation of liposomal particle pharmacokinetics was collected at E12.5, E14.5, and E16.5. Serum was collected by cardiocentesis after euthanasia from 4–6-month-old female mice. The assay samples were measured in triplicate.

Liposomal nanoparticle synthesis

Calcein-containing liposomal particles were prepared by Katayama Chemical (Osaka, Japan). Briefly, HSPC, cholesterol, and DSPE-PEG 2000 were dissolved in methanol/chloroform at the lipid mixture molar ratios of 57%, 38%, and 5%, respectively. The solvent was removed by heating at 37°C in a rotary evaporator. The lipid film was mixed with calcein solution at the molar ratio of 1:2.96 at 37°C for 1 h. The solvent was ultrasonicated for 30 min using a bath-type device. The solvent was transferred to Falcon tubes and freeze-thawed. Free calcein was removed by ultrafiltration (NMWL: 300,000). After ultrafiltration, the liposome solution was sterilized by filter filtration using a 0.22 μm filter.

Calcein release assay

Liposome nanoparticles were diluted 10-fold with the serum and amniotic fluid and incubated at 37°C. Samples were collected at 0 h, 6 h, 1 day, 3 days, 7 days, and 14 days after incubation. The samples were further diluted 100-fold with saline. The fluorescence intensity of the samples was measured at 490 nm in a 96-well plate. Liposomal calcein release was disrupted using 0.1% Triton X-100. The pharmacokinetics were expressed as the percentage of fluorescence intensity upon Triton X-100 addition, where 100 is the intensity at the time of addition. Percent calcein release was calculated using the following equation:

$$\text{Calcein release (\%)} = (F - F_0)/(F_{max} - F_0) \times 100$$

where F_0 and F represent the initial fluorescence and fluorescence at each time point, respectively, and F_{max} is fluorescence after Triton X-100 addition.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Significant between-group differences were determined using one-way ANOVA with Tukey HSD post-hoc test. All statistical analyses were performed using SPSS (IBM Version 28). In all tests, a $P < 0.05$ was considered significant.

RESULTS

Components of the embryo, placenta, and amniotic fluid

Embryo weight increased following a exponential trend and reached 1.240 ± 0.021 g at E17.5. Placental weight increased gradually from 0.043 ± 0.003 g at E11.5 to the maximum of 0.137 ± 0.003 g at E16.5. The volume of amniotic fluid increased from 0.065 ± 0.004 g on E11.5 to the maximum of 0.189 ± 0.007 g at E15.5, followed by a sharp decrease to 0.034 ± 0.007 g at E17.5 (Figure 1).

Liposomal particle pharmacokinetics

The mean nanoparticle size was 92.7 nm, lipid concentration was 17.30 mg/mL^{-1}, and zeta-potential was -25.9 mV. The nanoparticles contained 2.20 mM calcein. The calcein release rate gradually increased up to 14 days in both serum and amniotic fluid E12.5, E14.5, and E16.5. The release rate re-

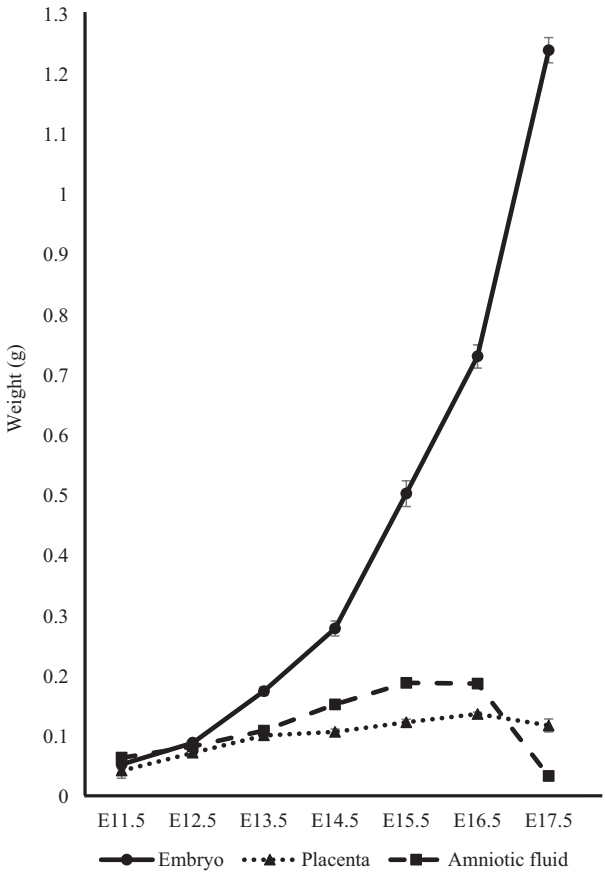

Figure 1. Embryo and placenta weight and amniotic fluid volume in Jcl/ICR mice between E11.5 and E17.5.

mained within 2.5% up to day 7, and the highest release rate was 3.92 ± 0.26% in the serum on day 14. There were no significant differences in the release rate between the amniotic fluid and serum at each point (Figure 2).

DISCUSSION

The present study revealed that the calcein release rate of liposomal nanoparticles in the amniotic fluid at different time points during gestation did not different from that in the serum for as long as 14 days. Drug carriers used in drug delivery systems require structural stability, as they must prevent leakage of the encapsulated drug to the target tissue in order to reduce side effects in non-target tissues. Our findings suggest that the encapsulated drug release rate of liposomal particles remains low in the amniotic fluid, indicating their suitability for stable drug delivery in the amniotic fluid.

In the present study, mice were used to observe the kinetics of liposomal particles in the amniotic fluid. The morphology of placenta varies across mammalian animal species [9]. The placenta of

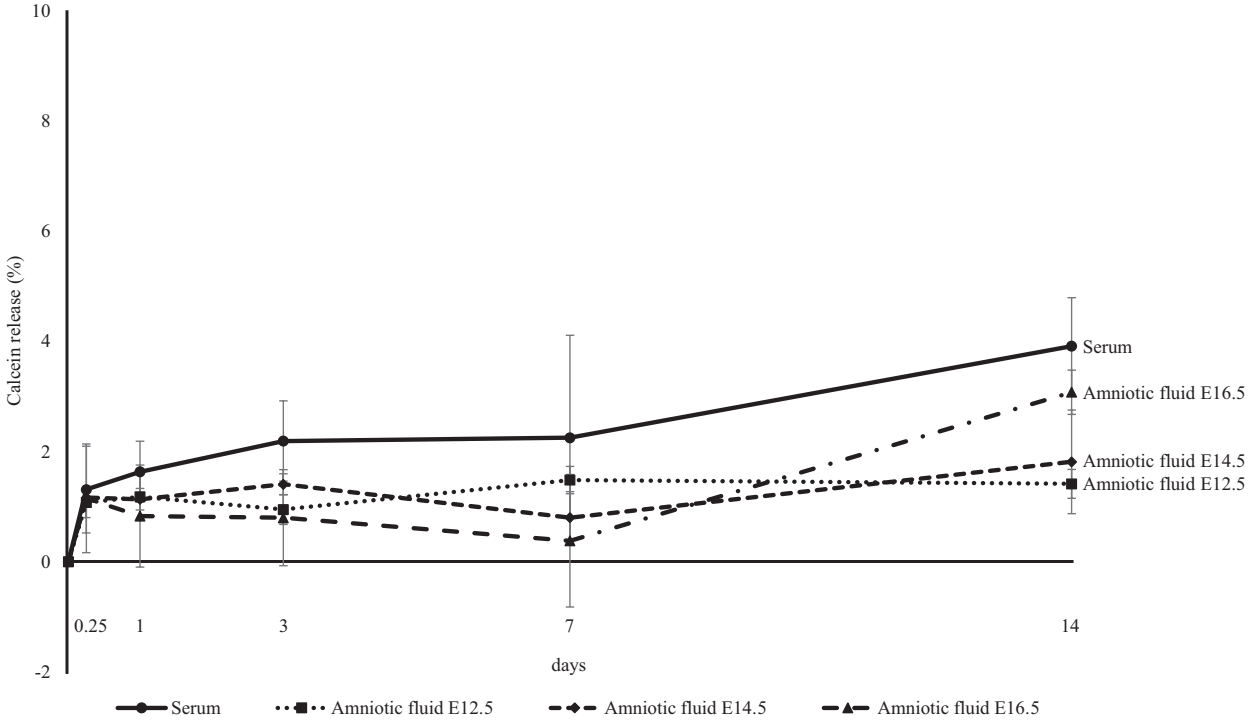

Figure 2. Calcein release rate in the serum and amniotic fluid. The kinetics are expressed as the percentage of fluorescence intensity at 490 nm when the liposomes were disrupted with Triton X-100 (fluorescence intensity = 100).

mice is hematogenous and structurally similar to that of humans [8], and mouse models are commonly used to study intrauterine drug delivery systems because of their short gestation period and a large number of animals that gestate at the same time [10,11]. Jcl/ICR mice, which have a relatively large body and high litter size, were used to collect amniotic fluid in this study. The total conceptus increases as pregnancy progresses, whereas the amount of amniotic fluid decreases towards the end of gestation [12]. The amniotic fluid volume is closely related to placenta and fetus, and thus, reflects their function [13]. The changes in placenta, fetus weight, and the amount of amniotic fluid in the Jcl/ICR strain were confirmed to be similar to other strains of mice, which is in agreement with existing reports [14].

In the present study, mouse serum was selected for the comparison of the stability of liposomal particles in the amniotic fluid. The stability of lipid-based nanoparticle systems in the serum has been reported in humans [15], bovine fetuses [16], and mice [17,18].

The volume and composition of the amniotic fluid changes dramatically during pregnancy. Several previous studies have reported amniotic fluid dynamics in mice [14,19], and JcL/ICR mice used in the present study showed similar amniotic fluid volume dynamics, with the maximum amniotic fluid volume at E16.5, followed by a sharp decrease. Changes in the composition of fetal amniotic fluid may affect the kinetics of liposomal particles. The osmolality of mouse amniotic fluid remains unchanged until before delivery, although the concentrations of electrolytes, such as sodium, potassium, calcium, and glucose, are altered [19]. In addition, protein concentrations in the amniotic fluid are lower than those in the serum [20]. In mice, the protein concentration in the amniotic fluid is nearly a-tenth of that in the serum [11]. In amniotic fluid with such a variable composition, the liposomal particles retained high encapsulated calcein between E12.5 and E16.5.

Amniotic fluid from E12.5 to E16.5 was used in the present experiments. In mouse fetuses, major organs are determined and protodifferentiated tissue starts to be characterized into fully differentiated cells at E12.5 [21]. Development of fetal lungs at E16 in mice marks the end of the canalicular stage, and the respiratory network becomes functional [22]. Intra-amniotic administration of liposomal particles during this periods can contribute to normal histogenesis of organs or therapeutic drug delivery to the fetal lungs via fetal breathing movements [11,23]. Our observation of the long-term stability of liposomal particles in the amniotic fluid at various time points extends the potential application of liposomal particles for therapeutic contribution.

Previous studies have reported that the administration of liposomal particles into the amniotic fluid reaches the fetal epidermis, dermis [24], lungs [10,11,25], intestinal epithelium [11,26], and liver [11]. The present experiment was *in vitro* and did not examine whether the liposomal particles reached the major organs. However, the liposomal particles used in the present study were smaller than 100 nm, which is the ideal size for nucleic acid delivery [27]. In addition, highly stable liposomal nanoparticles enable potent mRNA delivery [11]. Therefore the stable liposomal nanoparticles in the amniotic fluid in the present study are expected to enable drug delivery to the major organs via intrauterine administration. The stability assessment of liposomal particles based on size and zeta potential measurements as well as their *in vivo* kinetics and delivery to the major organs warrant further study in the future.

CONCLUSION

The present study showed that the kinetics of liposomal particles in the amniotic fluid showed similar internalization rates to those in the serum for as long as 14 days. Liposomal particles remain stable for prolonged periods in the amniotic fluid, the quantity and quality of which during pregnancy; therefore, liposomal particles are expected to contribute to fetal therapy, in which drugs are administered in the amniotic fluid.

Ethical approval

The study protocol was approved by the Ethics Committee for Animal Experimentation of Shimane University (approved numbers: IZ2-140). The mice were handled in accordance with the institutional

guidelines.

Author contribution

YA conceived the idea of the study. NO and NH contributed to data analysis. NO drafted the original manuscript. All authors approved the final version of the manuscript to be published.

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Confict of interest

None.

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