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ORIGINAL PAPER

Artificial induction of superfetation in the European hare (*Lepus europaeus*)

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Abstract The aim of this study was to induce superfetation in hares. On day 37 or 39 of pregnancy, female hares were treated with GnRH analogue and artificially inseminated with fresh semen. Ultrasonographic examination showed that pregnant females had follicles on the ovary on 37 days of pregnancy. After a few days from insemination performed during pregnancy, all females gave birth to healthy young, and one of the females inseminated on day 37 of pregnancy gave birth to two healthy young 42 days after insemination performed during pregnancy (39 days post first delivery). The same female in the next year gave birth to two healthy young 43 days after second insemination that was performed on the day 37 during pregnancy. The obtained results confirm that superfetation is possible to occur in hares.

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Introduction

European hare reproduction strategy is based on a relatively long breeding season (January–August). During this period, a female hare can give birth to four litters and an average litter size is 2–4 (Raczyński 1964). However, European hares inhabiting Mediterranean ecosystems can reproduce all over the year (Antoniou et al. 2008). Additionally, a phenomenon of superfetation (conception by an already pregnant female) may occur in a hare, which, according to Raczyński (1964), occurs very occasionally in the natural environment and does not have a greater influence on the effectiveness of the European hare reproduction. In general, superfetation occurs very occasionally in other animal species and is recognized as pathology (Gee 1971), whereas it is considered to be normal in the hare. Bloch et al. (1967) described presence of a foetus, nearly ready to be delivered, and fertilized ova (divided into four cells) in the uterus of a pregnant hare. Martinet (1980) showed that copulation occurred frequently in the hare from days 34 to 40 of pregnancy (the peak of sexual activity occurred on days 37 and 38 of pregnancy, i.e. 3–4 days before parturition), and copulation at this time induced ovulation resulting in the superfetation (Caillol and Martinet 1976). Martinet (1980) also showed that mating or treatment of hares with hCG during pregnancy induced ovulation. On the other hand, Stavy and Terkel (1992) maintained a breeding colony of paired hares in Israel for over 10 years, but no cases of superfetation were observed. Lincoln (1974) observed ovulation immediately following parturition or sometimes a short before it, but no females ovulated during early and middle phase of pregnancy and no

case of superfetation was found. As can be seen, the data on superfetation in hares are very different, and this subject has been recently reviewed in detail by Roellig et al. (2010b).

Therefore, the aim of this study was to induce the superfetation experimentally in hares by induction of ovulation in pregnant females with the use of GnRH analogue and insemination with fresh semen. In order to explain conditions that enable the induction of superfetation in the European hares, we used ultrasonographic examination of reproductive tract in female hares with the special attention on the ovary, i.e. if follicles are presented on the ovary during the late stage of pregnancy.

Materials and methods

Eleven adult females (body weight from 3.5 to 4.1 kg) and two adult males (body weight of 3.8 and 4.2 kg) were used in this experiment. During the experiment, the hares were housed in individual cages located in Wrocław (south-west Poland, 57°07′ N, 17°02′ E) and were exposed to natural daylight and ambient temperature. The hares were watered and fed ad libitum with a diet composed of hay, oat and a full-component mixture for hare. Additionally, branches from fruit and willow trees were given. In the middle part of February, all females were artificially inseminated. Just before artificial insemination (AI), the females were treated with a mixture of xylasin (Sedazin; Biowet Puławy, Poland) and ketamin (Bioketan; Biowet Puławy, Poland) (4 and 12 mg, respectively, per kilogramme of body weight, intramuscular injection). A dozen or so of minutes after the hares were immobilized, each female was then treated with a GnRH analogue (Receptal; Intervet International GmbH, 0.00168 mg of buserelin, intramuscular injection) for induction of ovulation as described before (Kozdrowski 2009; Kozdrowski et al. 2009; Kozdrowski and Siemieniuch 2009).

The semen for AI was collected by electroejaculation (Kozdrowski 2009; Kozdrowski et al. 2009). Males, before semen collection, were subjected to 12-h starvation and were then treated with a mixture of xylasin (Sedazin; Biowet Puławy, Poland) and ketamin (Bioketan; Biowet Puławy, Poland) (6 and 15 mg, respectively, per kilogramme of body weight, intramuscular injection). A few minutes after achieving full anaesthesia, semen was collected. After initial evaluation, the semen was diluted with the extender of the following composition: tris, 313.79 mM; citric acid, 103.07 mM and glucose, 33.3 mM (Roca et al. 2000). The diluted semen was used for AI within 1 h after dilution and each female received 1 ml of the diluted semen containing 30×10^6 spermatozoa with spermatozoa motility above 80% (Kozdrowski and Siemieniuch 2009). The diluted semen was slowly deposited with a plastic pipette introduced into the vagina as deep as it was possible (8–10 cm).

The inseminated females were divided into two groups. Six females included in group A were again anaesthetized with the use of the above-mentioned protocol on day 37 after AI (AI day 0). At least 12 min after the hares were immobilized, palpable examination for detection of pregnancy were performed. Immediately after the examination, the pregnant females were treated with the same dose of GnRH analogue as mentioned above and were inseminated again (second AI) with the same dose of fresh semen as mentioned above. Five females included in group B underwent the same procedures 2 days later, i.e. on day 39 after the first AI (AI day 0). Next, no procedures were performed on the females from groups A and B until delivery and for 7 weeks following the delivery. Every day in the morning (during feeding), the cages were checked for newborn hares. The young were separated from their mothers 4 weeks post-partum.

In the next year, seven adult females (all were used in the previous breeding season) constituted group C and one adult male (this male was also used in the previous breeding season) housed and fed in the same way as described above were included for observations. Females and semen for AI were prepared in the same way as described above, and first insemination was also performed in the middle part of February. In this group, all females were introduced for ultrasonographic examination on day 37 after first AI. Up to this time, only few papers described ultrasonographic examinations of reproductive tract in European hare females, and their authors focused on foetal growth (Hackländer et al. 2003; Hildebrandt et al. 2009; Roellig et al. 2010a) and also described ultrasonographic findings on the ovary during pregnancy and AI (Hildebrandt et al. 2009; Roellig et al. 2010a). Hares for USG examinations were positioned in dorsal recumbency; abdomen was shaved and covered with alcohol and gel. Transabdominal ultrasonographic examination was performed with the Ultrasonographic System Mindray M5 (Mindray Medical International Limited, China) equipped with a 8–12-MHz linear transducer. The ovaries were identified behind the caudal pole of the ipsilateral kidney. Just after examination, all pregnant females were again inseminated (second AI) with the same way as described above, and no procedures were performed on those females until delivery and for 7 weeks following the delivery. Every day in the morning (during feeding), the cages were checked for newborn hares. The young were separated from their mothers 4 weeks post-partum.

Results

On day 37 after the first AI, four females from group A were pregnant (including three females with single preg-

nancy and one female with twin pregnancy). On day 39, four female hares from group B were also pregnant (including two females with single pregnancy and two females with twin pregnancy). A pregnancy rate for the two groups together amounted to 73%.

Within group A, the female hares with single pregnancy gave birth to healthy young after 40, 41 and 42 days from the first AI (i.e. after 3, 4 and 5 days from the second AI, respectively). The female with twin pregnancy gave birth to healthy litter after 41 days from the first AI (i.e. 4 days from the second AI). Within group B, the female hares with single pregnancy gave birth to healthy litter after 40 and 43 days from the first AI (i.e. 1 and 4 days after the second AI), whereas the females with twin pregnancy gave birth to healthy litter after 41 and 42 days from the first AI (i.e. 2 and 3 days after the second AI).

On day 39 after the first delivery (42 days after the second AI), a female from group A gave birth to two healthy young (the first pregnancy of this female lasted for 40 days and the female gave birth to one healthy young). The remaining females from group A and group B did not give birth after the first delivery (after second AI).

On the day 37 after the first AI, four females from group C were pregnant (57% pregnancy rate). Ultrasonographic examination showed that all pregnant females had follicles on the ovary at this time (Fig. 1). All females from this group gave birth to one healthy young on 41 days after first AI. On day 39 after the first delivery (43 days after the second AI), one female from this group gave birth to two healthy young (second case of superfetation). However, it was the same female, which had successfully induced superfetation in the previous year. The remaining females from this group did not give birth after the first delivery (after second AI).

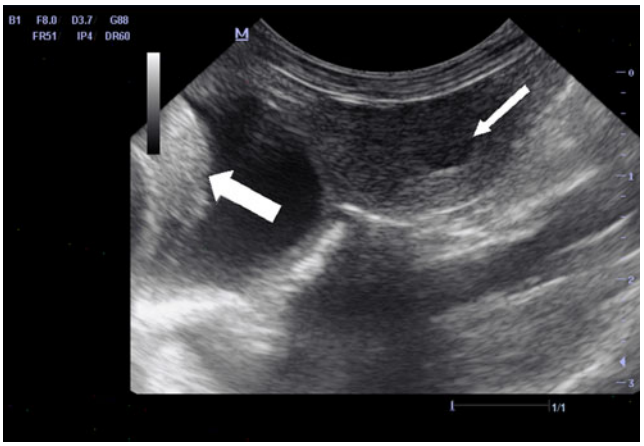

Fig. 1 Ultrasonography of the ovary with antral follicle (*narrow arrow*) and part of the foetus (*wide arrow*)

Discussion

During the breeding season, administration of hCG and GnRH analogues induce ovulation in hares (Caillol et al. 1986; Hildebrandt et al. 2009; Kozdrowski 2009; Kozdrowski et al. 2009; Kozdrowski and Siemieniuch 2009; Stavy and Terkel 1992). After the hormonal stimulation ovulation appears, the corpora lutea are formed and concentration of progesterone increased for about 2 weeks in those female hares that have not been mated or artificially inseminated (pseudopregnancy) (Caillol et al. 1986; Stavy et al. 1978). It was shown that after administration of GnRH analogue or hCG and insemination with the spermatozoa collected from epididymis or insemination with fresh or frozen/thawed semen, a percentage of pregnant females ranged between 50% and 80% (Hildebrandt et al. 2009; Kozdrowski 2009; Kozdrowski et al. 2009; Kozdrowski and Siemieniuch 2009; Stavy and Terkel 1992). Similarly, in this study, the percentage of pregnant females after the first insemination was 73% for group A and B and 57% for group C in the next breeding season. Out of 12 pregnancies found there, nine were single and three twin pregnancies, which also correspond to the numbers of young in early year pregnancies mentioned by most of the authors (Hackländer et al. 2003; Hildebrandt et al. 2009; Kozdrowski 2009; Kozdrowski et al. 2009; Kozdrowski and Siemieniuch 2009; Raczyński 1964).

We decided to carry out the next induction of ovulation and insemination on day 37 or 39 of pregnancy as, at this stage of pregnancy, the frequency of copulation between the hares considerably increases leading to ovulation and the next gestation (superfetation) (Caillol and Martinet 1976; Martinet 1980). Stavy and Terkel (1992), inducing superfetation by AI with spermatozoa collected from epididymis on day 38 of pregnancy, found abortion in only one case 22 days after insemination (19 days post-delivery). In our experiment, one female inseminated on day 37 of pregnancy gave birth to one healthy young after 3 days from the second insemination and the next two healthy young after the following 39 days (42 days from the second insemination). To the authors' knowledge, it is the first case of the induction of superfetation described.

In this study, the administration of GnRH analogue to the pregnant female hares and the AI procedure did not have a negative impact on the course of pregnancy as all young were born alive and were raised by their mothers until day 28 of their lives. Stavy and Terkel (1992) stated, in turn, that the mean gestation period is shortened from 45 to 41 days in those females for which an attempt to induce superfetation was made on day 38 of pregnancy, which resulted in a lower birth weight of the young and, in natural environment, a shortened period of gestation, resulting in

lower weight at birth, might be a disadvantage to the offspring. However, the length of gestation in our study lasted from 40 to 43 days (AI day 0), which is consistent with the earlier observations carried out in the same conditions on the female hares in which superfetation was not induced (the length of pregnancy was from 38 to 44 days) (Kozdrowski 2009; Kozdrowski et al. 2009; Kozdrowski and Siemieniuch 2009) and is consistent with the observations in another breeding centre in the Central Europe (Hildebrandt et al. 2009). However, Stavy and Terkel's (1992) study was carried out under different geographical conditions and in another hare, and perhaps the attempt to induce superfetation on day 38 of pregnancy in the hare population with an average length of gestation amounting to 45 days might have been made too early. In addition, the procedures (insemination, hCG administration and vaginal stimulation) performed with the attempt to induce superfetation resulted in shortening of the gestation period from 45 to 41 days, leading to early delivery (Stavy and Terkel 1992). On the other hand, Martinet (1980) stated that injection of hCG in hares carried out between days 14 and 28 of pregnancy caused abortion and after day 32 did not influence the length of pregnancy. On the basis of our observation, one can state that neither the AI nor the injection of GnRH analogue on the days 37 or 39 of pregnancy has a negative impact on the course of gestation, delivery and raising the offspring.

The study aimed to show whether it was possible to induce superfetation in hares, and this aim was achieved; however, we cannot say anything about the frequency of superfetation occurrence and its significance for the hare reproduction. Based on results obtained in this work, it seems that this phenomenon is rather rare because superfetation was successfully induced only two times and in the same female. It is also hard to say unambiguously what conditions must occur for superfetation to take place. However, it was shown that on 37 days of pregnancy, follicles are presented on the ovary. This study showed that single pregnancy is a factor that predisposes to the occurrence of superfetation and that the second AI should be performed 3 or 4 days before delivery. It can also be assumed that multiple pregnancy may also create favourable conditions for the occurrence of superfetation provided that foetuses are situated only on one side of the uterus. The obtained results confirm that superfetation is possible to occur in hares.

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References

Antoniou A, Kotoulas G, Magoulas A, Alves PC (2008) Evidence of autumn reproduction in female European hares (*Lepus europaeus*) from southern Europe. Eur J Wildl Res 54:581–587. doi:10.1007/s10344-008-0182-y

Bloch S, Hediger H, Lloyd HG, Müller C, Strauss S (1967) Beobachtungen zur Superfetation beim Feldhasen (*Lepus europaeus*). Z Jagdwiss 13:49–52. doi:10.1007/BF01902045

Caillol M, Martinet L (1976) Preliminary results on plasma progesterone levels during pregnancy and superfetation in the hare, *Lepus europaeus*. J Reprod Fertil 46:61–64. doi:10.1530/JRF.0.0460055

Caillol M, Meunier M, Mondain-Monval M, Simon P (1986) Seasonal variations in the pituitary response to LHRH in brown hare (*Lepus europaeus*). J Reprod Fertil 78:479–486. doi:10.1530/JRF.0.0780479

Gee CD (1971) A case of superfetation in the cow. Aust Vet J 47:179. doi:10.1111/j.1751-0813.1971.tb02138.x

Hackländer K, Miedler S, Beiglböck C, Zenker W (2003) The assessment of female reproduction and gestational age in European hares (*Lepus europaeus*) using ultrasonography. Mamm Biol 68:187–191. doi:10.1078/1616-5047-00082

Hildebrandt TB, Roellig K, Goeritz F, Fassbender M, Krieg R, Blottner S, Behr B, Hermes R (2009) Artificial insemination of captive European brown hares (*Lepus europaeus* PALLAS, 1778) with fresh and cryopreserved semen derived from free-ranging males. Theriogenology 72:1065–1072. doi:10.1016/j.theriogenology.2009.06.026

Kozdrowski R (2009) The effect of trehalose on post-thaw viability and fertility of European brown hare (*Lepus europaeus* Pallas, 1778) spermatozoa. Anim Reprod Sci 116:326–334. doi:10.1016/j.anireprosci.2009.02.012

Kozdrowski R, Siemieniuch M (2009) Inducing ovulation and artificial insemination in the European brown hare (*Lepus europaeus* Pallas, 1778). Anim Reprod Sci 112:377–383. doi:10.1016/j.anireprosci.2008.04.030

Kozdrowski R, Dubiel A, Noszczyk-Nowak A (2009) Comparison of acetamide and dimethylsulfoxide (DMSO) as cryoprotectants of European brown hare (*Lepus europaeus* Pallas, 1778) spermatozoa. J Reprod Dev 55:45–49. doi:10.1262/jrd.20085

Lincoln GA (1974) Reproduction and "March madness" in the brown hare, *Lepus europaeus*. J Zool Lond 174:1–14. doi:10.1111/j.1469-7998.1974.tb03140.x

Martinet L (1980) Oestrus behavior, follicular growth and ovulation during pregnancy in the hare (*Lepus europaeus*). J Reprod Fertil 59:441–445. doi:10.1530/JRF.0.0590441

Raczyński J (1964) Studies on the European hare. V. Reproduction. Acta Theriol 9:305–352

Roca J, Martinez S, Vázquez JM, Lucas X, Parrilla I, Martinez EA (2000) Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15°C. Anim Reprod Sci 64:103–112. doi:10.1016/S0378-4320(00)00185-8

Roellig K, Goeritz F, Hildebrandt B (2010a) Ultrasonographic characterization of prenatal development in European brown hares (*Lepus europaeus* PALLAS, 1778): an evolutionary approach. Reprod Fertil Dev 22:448–458. doi:10.1071/RD09098

Roellig K, Menzis BR, Hildebrandt B, Goeritz F (2010b) The concept of superfetation: a critical review on a 'myth' in mammalian reproduction. Biol Rev. doi:10.1111/j.1469-185X.2010.00135.x

Stavy M, Terkel J (1992) Interbirth interval and duration of pregnancy in hares. J Reprod Fertil 95:609–615. doi:10.1530/JRF.0.0950609

Stavy M, Terkel J, Kohen F (1978) Plasma progesterone levels during pregnancy and pseudopregnancy in the hare (*Lepus europaeus syriacus*). J Reprod Fertil 54:9–14. doi:10.1530/JRF.0.0540009