# Case Report Rapport de cas

## Acephalous lamb from an in vitro-produced sheep embryo

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**Abstract —** This is the first report of an acephalous lamb from the transfer of an in vitro-produced sheep embryo. Twelve in vitro-fertilized embryos were transferred to 4 recipient ewes (3 embryos/ewe). Two ewes remained pregnant: one delivered a normal female lamb, the other a male acephalous lamb. Possible contributing factors are discussed.

**Résumé — Agneau acéphale provenant d'un embryon de mouton produit in vitro.** Ceci constitue le premier rapport d'un agneau acéphale provenant du transfert d'un embryon de mouton produit in vitro. Douze embryons fertilisés in vitro ont été transférés à 4 brebis récipiendaires (3 embryons/brebis). Deux brebis sont restés gravides : une a mis bas à un agneau femelle en santé, l'autre à un agneau mâle acéphale. Les facteurs contributifs possibles sont discutés.

(Traduit par Isabelle Vallières)

Can Vet J 2009;50:501–505

Prepubertal sheep ovaries were collected during the nonbreeding season (June to July) from a slaughterhouse and transported to the laboratory in saline at 30°C to 35°C. A total of 4 ewes received 12 in vitro fertilized (IVF) embryos (3 embryos/ewe) that had been obtained by the harvest, culture, and fertilization of oocytes and the culture of blastocysts, with slight modifications of procedures previously described (1). The oocyte maturation and blastocyst formation rates were > 85% and 37%, respectively. Following 7 d in culture, grade 1 and 2 expanded blastocysts (2) were surgically transferred into the uterine horn of synchronized recipient ewes. Pregnancy was diagnosed by hormonal assay at 30 d and checked by ultrasonography at days 55 and 140. Three out of 4 recipients were pregnant on day 30; and 2 were still pregnant on days 55 and 148. Following cesarian section (on day 148), a normal female lamb (4.6 kg) was recovered from one recipient and a male acephalous lamb (3.9 kg) from the other. After delivery, the heartbeat in the acephalous lamb lasted for about 10 min.

## Case description

In the acephalous lamb, the hind and forelimbs had developed normally. Hair was absent from the entire body surface, with the exception of some parts of the neck and carpal joints. The cranial end of the neck ended in 2 adjoined ears without an orifice (Figures 1, 2).

On examination at necropsy, the skull was obviously undeveloped. After making an incision between the ears, an empty space into which the epiglottis and esophagus opened was observed. The esophagus, with an abnormally thin wall, resembling that of a megaesophagus, and abnormal mucosal membrane, was connected to the alimentary tract. The intestinal tract was completely formed, but it had an abnormally thin wall in which the muscular layer was absent. The lungs were uniformly red and atelectatic. The heart, which was extremely small and incompatible with the size and age of fetus, had 2 ventricles and 2 atria. The kidneys were present with a distinct medulla and cortex. The liver, without a gall bladder; other internal organs; and testes were present but underdeveloped. A whole-body radiograph showed normal formation of the skeleton, except for the skull, which appeared as a compact bony structure articulating with the atlas (Figure 3).

## Discussion

Acephalia, congenital absence of the head, is rarely reported in the ovine species. During a 3-year investigation, congenital defects of the nervous system were found in 1.5% of 4417 lambs necropsied. Twice as many male as female lambs were affected (3). Among neural tube defects (NTDs), anencephaly and spina bifida in humans and hydrocephalus and spina bifida in sheep are more common and are important factors in infant and fetal mortality (3–5). To date, in humans, few specific environmental causes of neural-tube defects have been recognized, except for relatively rare sources in utero exposure, such as maternal diabetes (5,6) and maternal use of some antiepileptic drugs, such as valproic acid (7). Other factors, including fever and hyperthermia in early pregnancy (8,9) and obesity (10,11), have been proposed. The extent to which occupational or environmental exposure may cause neural tube defects is still unclear. The genetic and environmental causes of neural tube defects also remain to be fully determined.

Based on retrospective studies in humans, there are controversial reports regarding the relationship between congenital malformation and infants born after IVF. A Danish registry study

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**Figure 1.** Acephalous male lamb; hair was absent from the entire body surface, with the exception of some parts of the neck and carpal joints.

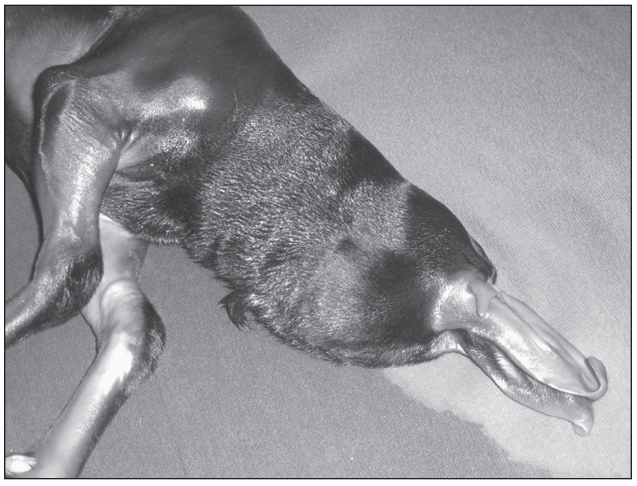

**Figure 2.** Two adjoined ears without an orifice connected to the neck.

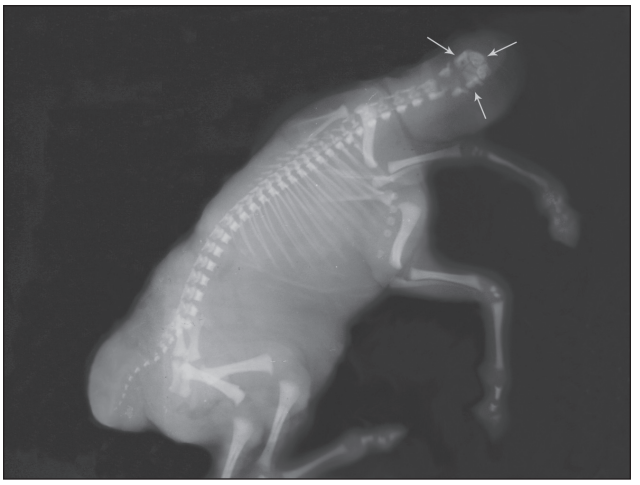

**Figure 3.** Lateral whole body radiograph. The atlas articulates with the nearly compact bony structure [the undeveloped skull (arrowheads)].

comparing outcome after IVF with matched controls found that 4.8% of IVF newborns and 4.6% of control newborns had a congenital malformation (12). However, there is evidence indicating that there is excess risk for congenital malformation, including NTDs, in infants born after IVF (13).

In animals, there is also a large body of evidence indicating the occurrence of various abnormalities following transfer of embryos from in vitro-production (IVP) systems (14–17). For instance, reported abnormalities in cattle following transfer of embryos from IVP systems have included lowered pregnancy rates; increased rates of abortion; production of oversized calves with altered organ development, altered energy metabolism, increased perinatal mortality, and musculoskeletal deformities and disproportionalities; and hydrallantois and other abnormalities of placental development (14–16). Collectively, these fetal characteristics were described as "large offspring syndrome"; they have also been identified in fetal and newborn lambs (18,19). Considering that in the in vitro-procedure, the oocyte and sperm and the embryos produced are exposed to many synthetic and natural compounds (serum, bovine serum albumin, hormones), the extent to which the gametes and embryos are adversely influenced by those compounds needs to be extensively investigated. It is already known that in sheep several abnormalities, including premature blastulation (20), accumulation of cytoplasmic "lipid-like" inclusions (21), and an increase in both gestation length and birth weight of lambs, occur following transfer of cultured embryos in the presence of human serum (20,22). In studies exposing in vivo-matured and -fertilized sheep zygotes to different culture systems, the expression of fetal oversize was dependent on both the serum source and the presence or absence of cocultured granulosa cells (23,24). There is also evidence indicating that different culture conditions can produce embryos with differing morphology, apparent chemical composition, and rate of development (22). There is, however, no direct evidence in respect to the effect of components of culture media on the production of an acephalous lamb.

In the present study, the procedure of in vitro-production, with slight modifications, was similar to what has been described previously (1). Therefore, the presence of complex sera (fetal calf serum, sheep serum) and putative agents, including growth factors, free radicals, ammonia, and steroids hormones, as perturbing factor(s) in culture media, might have influenced the normal events of embryo development at different stages of in vitro-production. In this context, comparisons of gene expression for bovine preimplantation embryos produced both in vivo and in vitro have demonstrated that expression of both imprinted and nonimprinted genes can be affected by in vitro embryo production (25–28). Compared with in vivo-produced control embryos, levels of messenger RNA (mRNA) expression in IVP embryos can differ with the stage of embryo development (25,26,28).

Alteration of epigenetic patterns associated with aberrant expression of imprinted genes during subsequent embryonic, fetal, and placental development in conceptuses and fetuses resulting from the transfer of IVP embryos have also been demonstrated (29–31). Although these observations support the role of altered expression of imprinted genes as a mechanism for the

large offspring syndrome, the additional observations of altered expression of nonimprinted genes in response to IVP in both preimplantation-stage embryos (27,32) and fetuses (33) point to the occurrence of a more generalized mechanism. Alternatively, they may indicate that in vitro culture and manipulations may influence methylation patterns that may be involved in the regulation of nonimprinted genes, as has been suggested in association with genetic disease (34). In this context, the Otx2 gene is thought to function in specifying territories in the rostral part of the brain and possibly the entire rostral part of the head of vertebrates. In mouse homozygous null mutants, all structures of the rostral part of the head are generally missing, and the embryos never survive beyond embryonic day 10 (35,36). Heterozygotes, however, exhibit a wide range of phenotypes from complete acephaly to relatively minor skeletal and neural defects (35). In the current report, though no genetic analysis was carried out, the probability that predisposing factors could exert an effect on embryo development through alteration in gene (s) function has to be kept in mind.

Another contributing factor to the quality of embryos produced in vitro is oocyte aging. There is a report of teratogenic effects of postovulatory oocyte aging to microcephaly and acephaly in fish and amphibian species (37). Since, in the current study, the aspirated oocytes for in vitro embryo production were mostly in the germinal vesicle stage, oocyte aging was excluded from the list of contributing factors. Indeed, after 24 h of oocyte maturation, the oocytes were immediately exposed to the sperm, hence there was little chance for oocyte aging.

There are also numerous post embryo transfer and post implantation factors in the etiology of NTDs reported in several species. In humans, it is believed that the mother's diet and vitamin intake may play a role in NTDs. The risk estimates were lowest for women whose diets were rich in choline, betaine, methionine, and folic acid (38). Other nutrients and nutrition-related factors have also been observed to influence the risk of NTDs (39–41), and increased intakes of sugar (42), maternal diabetes (6), maternal hyperinsulinemia (43), and prepregnancy obesity (11,44) have been associated with elevated risks of NTDs. Increased intakes of sugar might influence the risk of NTDs through a common pathway of altered glycemic control and insulin demand. Thus, at the time of neural tube closure, embryos could theoretically receive excess glucose from the mother and be unable to regulate the excess. Elevated glucose concentrations in experimental systems have led to oxidative stress and embryonic depletion of inositol (45), and the latter has been implicated in abnormal closure of the developing neural tube in experimental studies (46,47). It has been also documented that nutritional status has a correlation with embryo survival and is a key factor influencing efficiency in assisted reproductive technologies (48,49). Conflicting results have been reported for the effects of low or high energy diets on oocyte quality and early embryonic development in ruminants, including sheep (50,51).

In the current study, the recipient ewes, aged 2.5 to 4 y, of similar body weight (47.5, $S_{\bar{x}}$ = 2.5 kg) and body condition score (3.4, $S_{\bar{x}}$ = 0.6) were used (1 = extremely thin to 5 = extremely fat). Their nutritional diet was adjusted with the stage of their pregnancy based on National Research Council guidelines (52). Therefore, the nutritional status of the recipients should not have been a factor in the occurrence of this abnormality (acephaly).

In humans, most cases of acephaly have been associated with monochorionic twin pregnancy; reversed-arterial-perfusion sequence is a severe complication of such pregnancies and is characterized by the hemodynamic dependence of a "recipient" twin on a "pump" twin with the recipient twin exhibiting lethal abnormalities, including acardia and acephaly (53). While, in the current report, the pregnancy was singleton and a heart, despite of its quite small size, was present. Therefore, it seems the entity of this abnormality is different in humans from that reported herein for sheep. However, there has been no previous report on the occurrence of acephaly following transfer of IVP embryos in either humans or sheep.

Among numerous post-embryo transfer and post-implantation factors in the etiology of NTDs, hyperthermia could be another candidate. As reported, hyperthermia was the first teratogen in animals that was subsequently proven to be teratogenic in humans. Central nervous system defects appear to be the most common consequence of hyperthermia in all species, and cell death or delay in proliferation of neuroblasts is believed to be a major explanation for these effects (54). The range of NTDs induced by hyperthermia in experimental animals includes the following: anencephaly/exencephaly, encephalocele (55), and micrencephaly (54). Such defects have also been induced by heat in a variety of mammals, including guinea pigs, hamsters, rats, mice, rabbits, sheep, pigs, monkeys, and humans (56). Since, in the current study, the early stages of embryo development coincided with hot summer months, it is possible that the embryo's development could have been negatively influenced by heat stress.

In conclusion, because of considerable variation in predisposing and contributing factors on the production of the acephalous lamb, it is impossible to define the specific cause or causes.

## Authors' contributions

Dr. Shirazi was involved with the in vitro production of the embryos, the embryo transfer, the ultrasonography, and the necropsy, and wrote the manuscript. Drs. Ahmadi, Heidari, and Shams-Esfandabadi were involved with the in vitro production of the embryos, the embryo transfer, and the preparation of the manuscript. Dr. Jadidi was involved with the embryo transfer, the ultrasonography, and the preparation of the manuscript.

## Acknowledgments

The authors thank the Lori-Bakhtiari sheep breeding center (Shooli station), the personnel in the Department of Pathology, Faculty of Veterinary Medicine, Shahrekord University, and Najafabad's slaughterhouse staff for their cooperation. CVJ

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