Deaths of Transgenic Calves at AgResearch's Ruakura Facility

Report to Minister of Research, Science and Technology

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Review team

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Executive summary

Background

- Two transgenic calves carrying a human follitropin (FSH) gene died unexpectedly in February 2009 at AgResearch's Ruakura facility. A third animal showing the same physical signs was euthanased. A fourth animal did not show the same signs and remains alive and healthy.
- The study was part of a project to develop transgenic cattle that produce human FSH as a biopharmaceutical in their milk.
- FSH is a hormone that controls human gonadal function. It is used clinically in the treatment of infertility. A recognised risk of treatment with FSH is a syndrome of ovarian enlargement and hyperactivity.
- Screening of candidate proteins according to commercial (for example, market size) and technical (for example, potential adverse effects on the host animal) criteria led to AgResearch's identification of human FSH as a target for transgenic expression in cattle.

Experiment

- The human FSH transgene introduced into the calves was designed to become active specifically in the mammary gland and only during lactation, avoiding the effects of systemic secretion of this highly active hormone. The scientific principle of this targeted approach had been confirmed by earlier studies in mice and cattle.
- Unexpectedly, the FSH transgene behaved differently than predicted from the earlier studies. Although expression of FSH was generally confined to mammary gland tissue, the transgene became activated in the non-lactating prepubertal calves causing high systemic levels of FSH from early life. In three of the four calves, this led to greatly enlarged ovaries which precipitated the sudden death of two of the animals and the decision to euthanase the third.

Findings

- The necessary legal and ethical approvals for the study had been obtained as required by the Hazardous Substances and New Organisms Act 1996 and the Animal Welfare Act 1999.
- We find the identification of human FSH as an early target for AgResearch's transgenics programme to be puzzling. First, recombinant human FSH was already available for clinical use from two pharmaceutical companies, although AgResearch have shared with us a market analysis supporting the commercial rationale for their choice. Secondly, FSH is a highly bioactive molecule. It seems not unreasonable to us that the known occurrence of ovarian hyperstimulation syndrome in women receiving treatment with FSH, and of ovarian tumours in mice carrying an FSH

transgene, should have been taken into account during risk assessment for this study and then in the experimental design, even though the construct was designed to avoid this possibility.

- We find that decision-making during the course of the experiment would have been facilitated had AgResearch been able to perform prompt on-site measurement of blood hormone levels.
- We are confident that, subject to the caveat on measurement of blood hormone levels, the animals were managed appropriately throughout their lives and received a high standard of veterinary care. In particular, we note that the three calves with enlarged ovaries were behaving and grazing normally until their deaths.
- Finally, although the experiment produced unexpected results, procedures were performed correctly and outcomes (including successful production of FSH in the milk of the surviving animal) were documented accurately. We would therefore be reluctant to conclude that the experiment 'went wrong'. Rather, as is often the case in science, there were unexpected findings that suggest avenues for further research.

1. Background

Two transgenic cows carrying a human FSH gene died unexpectedly in February 2009 at AgResearch's Ruakura facility. A third animal showing the same physical signs was euthanased. A fourth animal carrying the same gene did not show the same signs and remains alive and healthy.

Media attention in May 2010 led to questions in the House and the Prime Minister's Chief Science Advisor was asked by the Minister for Research, Science and Technology to conduct an inquiry. Given Sir Peter Gluckman's scientific association with AgResearch, albeit in unrelated areas, he asked his Chief of Staff, Dr Alan Beedle, to undertake the report, assisted by Dr Jim Watson, an eminent New Zealand biotechnologist.

2. Process

We generated a structured set of questions for AgResearch which were answered by correspondence with [redacted], and [redacted]. We then conducted an interview with the following staff members from [redacted]

- [redacted]
- [redacted]
- [redacted]

Further questions arising from this interview were answered by correspondence. The project leader for this particular study ([redacted]) was overseas at the time of our inquiry, but was able to answer specific queries by email. We thank AgResearch staff for their cooperation during our inquiry.

AgResearch have had the opportunity to comment on this report.

3. Legislative situation

At the time of the matters under investigation, AgResearch Ltd had approval from the Environmental Risk Management Authority (ERMA) to develop transgenic cattle that can express functional therapeutic foreign (meaning from species other than cattle) proteins in their milk and to develop transgenic cattle to study gene function and genetic performance (application GMD02028).

The production and maintenance of genetically modified cattle, including the approval of specific experimental procedures, is monitored by the Ruakura Animal Ethics committee (RAEC). The committee ensures adherence to the AgResearch Ltd Code of Ethical Conduct for the use of Animals in Research, Testing and Teaching. The committee is appointed by

[redacted]), and consists at the minimum of:

- A senior member of AgResearch staff
- A veterinarian nominated by the New Zealand Veterinary Association who is not affiliated with AgResearch
- A nominee of the Royal New Zealand Society for the Prevention of Cruelty to Animals who is neither affiliated with AgResearch nor involved in the use of animals for research, testing or teaching
- A nominee of a territorial authority, Federated Farmers or regional council (in this case Environment Waikato) who is not affiliated with AgResearch, the scientific community or any animal welfare agency.

Additionally, the RAEC may include:

- An AgResearch veterinarian
- A member of AgResearch staff responsible for the procurement, production and maintenance of the animals
- A biometrician capable of assessing the statistical validity of an experiment; and
- Additional supernumerary members.

The initial animal ethics committee approval for this study (AE 11687) was obtained in November 2005. It expired in November 2008 at which time the first successful pregnancies had produced offspring and a revised proposal valid for 3 months was presented in November 2008 and a new application in February 2009.

4. Rationale for study

The commercial rationale for AgResearch's work on expressing foreign (generally human) proteins in ruminant milk is the production of high-value therapeutic proteins. Protein-based medicines such as hormones and monoclonal antibodies are usually expensive (thousands of dollars per course). Human-specific proteins can be produced by cell culture methods (although yields are low), but transgenic animal technology offers the potential for high yields and simple isolation from milk.

Issues to be considered when selecting protein targets for transgenic expression include the commercial value and ease of clinical development of the resulting protein, as well as the potential for harm to the transgene-bearing animal resulting from the biological activity of the protein within the animal.

In the experiment under consideration, the specific target protein was human follicle-stimulating protein (FSH or follitropin). FSH is produced by the pituitary gland and acts on the gonads to control reproductive function. FSH stimulates maturation of ovarian follicles (in

females) and spermatogenesis (in males) and is widely used in the treatment of infertility. Recombinant human FSH is commercially available but expensive (for example, treatment to induce ovulation in an infertile woman would cost at least $2000-3000 per cycle). The production of larger amounts of FSH by transgenic technology was considered by AgResearch's market analysis to be potentially commercially attractive.

It is well known that use of FSH in fertility treatment in women is associated with the risk of ovarian hyperstimulation syndrome. This is characterised by ovarian enlargement, abdominal discomfort and intra-abdominal fluid accumulation. It is occasionally severe and potentially fatal.

5. Experimental procedure

The approach used was to introduce the DNA sequence coding for human FSH into the nucleus of a bovine fibroblast (muscle cell). The nucleus from the fibroblast was then transferred into a cow oocyte (egg cell) from which the nucleus had been removed. The oocyte containing the donor nucleus will then begin to divide and develop into a fetus under the control of the genetic instructions from the donor nucleus, and the resulting embryo is implanted into the uterus of a 'surrogate mother' cow. This process, termed somatic cell nuclear transfer followed by embryo transfer, is well established as a method of producing transgenic animals but is fairly inefficient – 66 transfers of transgenic embryos were required to produce the five live animals used in this study (one control cloned animal and four carrying the FSH gene).

Normally, FSH is composed of two protein chains, coded on different chromosomes and each biologically inactive alone, that are synthesised separately in pituitary cells and then bind together to make the active hormone. The DNA sequence transferred into the bovine cells contained the sequences for the two protein chains fused together (a so-called 'tethered' construct). To attempt to ensure that the FSH was only expressed in the mammary gland and during lactation, a promoter sequence (or 'switch') taken from the bovine beta-casein (milk protein) gene was added to the inserted DNA sequence. Thus the experimental premise was that the transgenic system would become active only upon either natural or induced lactation and would be confined to the active mammary gland.

Previous studies by AgResearch showed that the 'tethered' two-chain human FSH molecule is biologically active and therefore would have the potential to cause adverse effects in animals carrying the transgene unless its expression was tightly controlled. A Korean study published in 2006 showed that expression of a similar construct in transgenic mice, also under the control of the beta-casein promoter to ensure mammary-specific expression, caused appearance of FSH in the serum of the animals because of 'leakage' of the protein from the mammary gland into the blood. Some of those mice with high expression of FSH in blood developed tumours in their reproductive organs. To ensure that transgene expression was truly controlled by the milk protein promoter, an 'insulator' sequence (a well-established method of preventing a gene being switched on by an unrelated promoter) was added to the construct used by AgResearch.

6. Timeline of the experiment

The five female calves relevant to this study were all born on 10 September 2008. Animals 08-030, 08-032, 08-033 and 08-034 all carried the human FSH transgene. Additionally, a clone 'sister' calf (08-031) that did not carry the FSH transgene was monitored as a control animal. All the FSH calves appeared normal at birth, but at 2 weeks of age began to show an unusually muscular or male-like appearance. They grew faster than the control animal and also appeared to have enlarged abdomens, but otherwise behaved and ate normally. All underwent regular blood sampling and their haematology screens were generally normal. In December 2008, animal 08-033 gave cause for some concern as it had a particularly large abdomen and appeared to have mild respiratory difficulty. It was assessed clinically on 22 December 2008 and again on 5 January 2009, at which time it was reported to have an awkward gait with the hind legs, and back bowing in the mid lumbar region. There was some fluid in the abdomen and some degree of precocious udder development, but no other abnormalities.

Hormone levels from the blood sampling were available in early 2009. Earlier measurement of hormone levels was not possible because of (i) the need to batch samples for analysis, (ii) the assays had to be performed off-site and (iii) the containment procedures required for samples from transgenic animals made it impractical to send multiple batches of samples for analysis. The hormone assays revealed very high circulating levels of human FSH (indicating that the FSH transgene was active in the pre-pubertal non-lactating calves) in the four FSH transgenic animals. Additionally, three of the four animals (08-030, 08-032 and 08-033) had high circulating levels of estradiol (a steroid hormone produced by ovarian follicles, indicating that the ovaries of these prepubertal animals had been activated by the high circulating levels of FSH).

The ovaries of the five calves were imaged by transvaginal ultrasound on 29 January 2009. Three of the transgenic calves (those with high circulating estradiol) showed abnormally large ovaries. At this stage of maturity, the normal bovine ovary is the size of a marble; the ovaries in these animals were the size of golf balls or tennis balls. It is impractical to perform transvaginal ultrasound on younger animals because of the size of the probe, and larger imaging facilities are not available at Ruakura.

In spite of these issues, the calves were eating normally and gaining weight. They were being monitored for appearance and behaviour at least twice daily.

On 15 February 2009, one of the animals with enlarged ovaries (08-030) was found dead in the paddock. Autopsy revealed a fatal haemorrhage from the uterine artery, presumably caused by the stretching and distortion caused by the enlarged ovaries.

Euthanasia of the remaining two animals with enlarged ovaries was considered at this time, but they were grazing normally and in good health (apart from the abnormalities described above) so this course of action was not chosen.

On 20 February 2009, animal 08-033 was also found dead in the paddock. Autopsy the same day showed that one of her enlarged ovaries had become twisted and separated from the uterus, which would have caused sudden death.

Euthanasia of the remaining animal with enlarged ovaries (08-032) was performed immediately. Analysis of tissues showed strong expression of the FSH transgene in the non-lactating mammary gland of this animal and weak expression in the lung.

The remaining animal (08-034) remains alive and healthy, although has chronically cystic ovaries because of her high circulating levels of FSH. She has been induced into lactation and produced high levels of FSH in her milk. It is unclear why this animal did not respond in the same way as the other calves to high serum FSH.

7. Specific issues of concern

(a) Was the aim of the experiment justified?

The production of large amounts of high-value therapeutic proteins by use of innovative biotechnological procedures is a public good and a commercially valuable outcome. The use of transgenic livestock that secrete the required proteins into their milk is an established approach that has led to regulatory approval of a human therapeutic in the US and Europe. AgResearch claim that FSH was selected as a target protein for production by this methodology on the basis of technical and commercial criteria, since FSH is a complex protein that in their hands has proved difficult to produce efficiently by more conventional biotechnological methods such as fermentation in micro-organisms. The aim of the present experiment was proof-of-concept testing of the expression of the human FSH transgene in the bovine mammary gland and the amount of FSH protein produced in milk, which is a necessary step in the development of a commercial product. Nevertheless, recombinant human FSH is already commercially available from two different pharmaceutical companies. Moreover, FSH is a highly biologically active molecule that when present in excess is known to be detrimental to humans and to animals. We therefore question the rationale that led to the choice of FSH as a target protein.

(b) Were the necessary legal and ethical procedures followed?

AgResearch Ltd had approval from ERMA to develop transgenic cattle that can express functional therapeutic foreign proteins in their milk and to develop transgenic cattle to study gene function and genetic performance (application GMD02028). The application was publicly notified and numerous submissions were received. As required by the Animal Welfare Act 1999, specific animal welfare issues arising from the approval were dealt with by the Ruakura Animal Ethics Committee. We have examined the relevant application (11687) to the RAEC and subsequent modifications, and are satisfied that animal ethics procedures were followed correctly.

(c) Were all reasonably anticipated scientific issues in inserting human FSH genes into cattle considered?

AgResearch had considered the potential problems of expressing biologically active proteins in the mammary gland, and had eliminated from their list of potential targets those that could cause local inflammation in the gland (such as immune-stimulating cytokines). The

experimental system that AgResearch uses is designed for specific expression of the target protein in the lactating mammary gland of sexually mature animals, and expression in pre-pubertal animals was not anticipated. Their previous experiments in transgenic mice and cattle had shown this expectation to be the case for proteins of low biological activity such as milk proteins, where 'leakage' of small amounts of the transgenic protein from the mammary gland into the circulation would be difficult to detect but would have little biological consequence. However, even low levels of expression of a highly biologically active protein may be detrimental to the host animal if that protein leaks into the circulation.

AgResearch informed us that they had considered the effects of leakage of FSH into the circulation of their transgenic animals during expression of the protein during lactation. The worst case scenario identified was apparently infertility of the animals, although the occurrence of ovarian hyperstimulation syndrome in women treated with gonadotropins has been known in the medical community since the 1970s. At the time of the initial ethics application for this work in November 2005, the Korean paper reporting ovarian abnormalities in mice transgenic for human FSH had not yet appeared (it would have been available in November 2006). However, the pregnancies that resulted in the transgenic calves under consideration would have been initiated by embryo transfer in late 2007, by which time AgResearch should have been aware of the Korean paper. We consider that AgResearch's risk assessment for this study should have been more alert to the possibility of deleterious effects of FSH in their transgenic cattle.

(d) Were the animals managed correctly after phenotypic changes were seen in early life, and after the death of the first animal?

The female FSH transgenic calves developed unusual muscularity and accelerated growth early in life. This 'masculinisation' was believed to be an effect of an excess of steroid hormone, probably as a result of cystic ovaries. This, together with the known effects of FSH, correctly prompted concern about the animals' hormonal profile and blood sampling for hormone assays and additional veterinary monitoring were instituted.

Monitoring of FSH activity can be done by (i) measuring blood concentrations of the hormone itself and/or of the steroid hormone estradiol, which is produced by the ovaries when they are stimulated by FSH, and (ii) examination of ovarian size and structure by transvaginal ultrasound imaging and direct rectal palpation. Although regular blood sampling was initiated in October 2008, within the first month of life of these animals, the results from these early samples were not available until early 2009. The reason for this delay was partly scientific and partly procedural. Valid comparison of the multiple samples obtained in late 2008 necessitated that all be analysed at the same time for standardisation. Moreover, the Ruakura facility does not have the capability to perform these hormonal assays and they had to be sent to an independent laboratory off-site. Containment requirements under ERMA regulations are such that sending samples from transgenic animals offsite is administratively complex, again meaning that it was decided to send a single batch of samples rather than multiple single samples.

With regard to veterinary examination of ovarian size and structure, we are advised that it is impractical and unethical to perform these procedures on young calves. The first physical examination of the ovarian state of these animals was performed at the earliest possible opportunity after the availability of the blood sample results.

After the availability of the blood sample results and the results of the clinical examination of the ovaries, it was clear that unexpected expression of the FSH transgene was occurring and leading to high systemic expression of the protein. However, in spite of some mild physical abnormalities the animals were not in distress and were grazing normally. The situation was re-assessed after the death of the first animal, and euthanasia of the other two calves with enlarged ovaries was considered at the time but rejected because of veterinary advice. After the death of the second animal, the remaining animal with enlarged ovaries was euthanased immediately.

We have no criticism of AgResearch in this respect. It is our opinion that the animals were managed correctly given the information available and their lack of distress. We would recommend, however, that if further studies with biologically active transgenic proteins are envisaged then consideration should be given to developing an in-house capability to monitor the systemic expression of the proteins on an ongoing basis. Regulatory attention might also be paid to removing the administrative burden around transportation of such low-risk samples, which has no scientific basis.

(e) Did the animals receive appropriate veterinary care?

The level of veterinary care at the Ruakura facility as described in the animal ethics applications we have examined is considerably superior to that in normal farm management. After the death of the first animal, the surviving calves were observed formally at least twice daily, and their clinical condition and welfare were constantly re-assessed. We have no criticisms of AgResearch in this respect.

8. Conclusion: did the experiment 'go wrong'?

It is a truism in science that it is not an experiment if the outcome is already known. The outcome of this study was unexpected in that the FSH transgene construct behaved differently than predicted from earlier mouse and cattle studies, leading to systemic expression of FSH protein in non-lactating prepubertal animals with effects on the ovaries of three of the four animals that were incompatible with long-term survival. Nevertheless, the calf whose ovaries did not respond strongly to high systemic FSH levels has survived, is generally healthy, and has produced large quantities of biologically active human FSH in her milk. In that respect, the experiment was successful. The consequences of the unexpected behaviour of the FSH transgene for the health of the transgenic animals were managed correctly with high regard for the welfare of the animals. We would therefore be reluctant to comment that the experiment 'went wrong'. Rather, as is often the case in science, there were unexpected findings that suggest avenues for further research.