The use of fetal bovine serum: ethical or scientific problem?

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Summary - Fetal bovine serum (FBS) is a common component of animal cell culture media. FBS is harvested from bovine fetuses taken from pregnant cows during slaughter. FBS is commonly harvested by means of a cardiac puncture without any form of anaesthesia. Fetuses are likely exposed to pain and/or discomfort and therefore current practice of fetal blood harvest is inhumane. Apart from moral concerns, several scientific and technical problems exist regarding the application of FBS in cell culture. Efforts should be made to reduce and preferably replace FBS by synthetic alternatives.

Key words: animal welfare, cell culture, fetal bovine serum, fetal pain, reduction, refinement, replacement, defined culture media.

Introduction

The technique of *in vitro* cell, tissue and organ culture is of great importance to science, medicine and industry. Amongst others, it is considered a major replacement alternative in animal experimentation (1, 2). Clearly, not every *in vitro* cell culture experiment or production method is an alternative to an animal experiment *per se* (1). Developments as *in vitro* fertilization, cloning and the creation of genetically modified animals rely on cell culture (3). To culture cells *in vitro*, culture medium is added to the cells for nurture. Usually, the medium contains fetal bovine serum (FBS) as FBS contains basic components, such as hormones and growth factors. Serum is blood without cells, platelets and clotting factors. Animal welfare concerns have been expressed concerning the methods employed in fetal bovine blood harvesting (4-6). This raises questions about the value of *in vitro* techniques as replacement alternatives. The aim of this paper is to describe the procedures used in fetal blood collection, and to regard them from an ethical and scientific point of view.

Materials and Methods

Information is based upon literature study and responses to questionnaires. Information on FBS harvesting methods was obtained by sending questionnaires to a variety of institutions in Europe, North, Central and South America, Southern Africa and Australia/New Zealand: Embassies and ministries covering agriculture and/or animal experimentation (77 entities contacted), animal welfare and animal rights organisations (190 organisations), commercial companies harvesting FBS (12 companies), commercial companies supplying FBS to end-consumers (33 companies), research institutes and universities (76 entities). These institutions were chosen as they were expected to have knowledge on FBS production methods, or to

have contacts with such knowledge. Their contact addresses were obtained mostly from the World Wide Web. The questionnaires contained standard questions on whether FBS harvest takes place at all in the country of the entity addressed, and the procedures employed in FBS harvest, as well as the volumes of blood and serum obtained from a bovine fetus. If technical information was provided by a given entity, follow-up questionnaires were sometimes sent to gain further insights. Information concerning pain in mammal fetuses, their resistance to lack of oxygen, problems related to the use of FBS in cell culture, and alternatives to FBS was obtained from the literature.

Results

Collecting factual data on fetal bovine blood collection proved more difficult than expected. Overall response was 61%. Of the 388 contacted entities, 14 provided technical information: 9 serum harvesters, 1 serum supplier, 3 ministry departments and 1 research institute staff member (a former USDA-inspector). Four suppliers and 1 harvester explicitly stated they consider detailed information relating to the methods used for FBS-harvest to be confidential.

Origin of bovine fetuses

The bovine fetuses from which blood is drawn for (commercial) FBS production are obtained from pregnant cows which are sent to slaughter for reasons such as crippling lameness, or when slaughtering herds of extensively kept beef cattle. Cows and bulls roam free in such herds, as a result of which several cows are pregnant at the time of slaughter (7).

Harvesting fetal blood

Bovine fetal blood is commonly harvested by cardiac puncture, because it minimizes the danger of serum contaminations with micro-organisms from the fetus itself, and its environment. The procedure requires specially trained staff. Fetuses should be at least 3 months old; otherwise the heart is too small for puncture.

The general procedure of a cardiac puncture is the following (see diagram). At the time of slaughter, the cow is found to be pregnant during evisceration (removal of the internal organs in the thorax and abdomen during processing of the slaughtered cow). The reproductive tract is removed from the carcass, and is dropped down a special stainless steel chute leading to the calf processing area, a room that is separated from the rest of the abattoir floor. The calf is removed quickly from the uterus and the umbilical cord is tied off, the fetus is cleaned from amniotic fluid, and is disinfected.

A cardiac puncture is performed by inserting a needle between the ribs directly into the heart of the unanaesthesised fetus and blood is extracted under vacuum into a sterile blood collection bag via a tube. In the absence of a vacuum pump, fetal blood may be obtained by means of gravity or massage. In this case the blood collection bag is placed at a level below the fetus. Once the blood has been obtained, it is allowed to clot at low temperature, after which the clotted substance is separated from the serum by refrigerated centrifugation. The fetus is processed for animal feed and extraction of specific substances like fats and proteins, among other things. A much less common technique is umbilical cord puncture.

The time that elapses between death of the mother cow as part of the regular slaughter procedure and the puncture is found to be either about 5 minutes (2 harvesters, 1 scientist) or about 25-30 minutes (1 harvester, 2 ministry departments). One harvester stated the ideal time is less than 30 minutes. The procedure of bleeding itself lasts about 2 - 5 minutes, depending

on the age of the fetus and the equipment. Hence, a bleeding procedure is ended about 5-10 or 25-35 minutes after death of the mother.

The amount of serum that is obtained depends on the size of the fetus, and thus on its age. As a rough estimate, about 50 % of serum remains after clotting. A bovine fetus of 3 months yields about 150 ml of raw FBS, at 6 months 350 ml and at 9 months (near-term) 550 ml. The global production per year of raw FBS is estimated to be around 500,000 litres (8-11). Hence, the number of bovine fetuses harvested annually should be greater than 1,000,000. A number of 2,000,000 has been mentioned (8).

Since the fetus is expected to be alive during blood collection, its possible suffering is considered. The described procedure may cause pain in the fetus, thus raising ethical questions. First, literature on the resistance of fetuses to lack of oxygen is discussed. The bovine fetus experiences anoxia, acute lack of oxygen, since oxygen-rich blood supply to the placenta ceases upon death of the maternal animal. Lack of oxygen may interfere with neural processes such as transmission of stimuli, and eventually leads to death.

Resistance of mammal fetuses to anoxia

Mammal fetuses and neonates show a remarkable resistance to anoxia, surviving anoxia longer, compared to adults of the same species (12-31). For example, adult rabbits survive for 1.5 minutes in pure gaseous nitrogen while pre-term rabbit pups at 29 days of gestation survive on average for 44 minutes, at 21 °C (13). Despite the fact that such data have been generated for a diversity of species, including the underlying biological mechanisms, no such study involving bovine (preterm) neonates was found.

Permanent brain damage occurs if anoxia is maintained longer then the time to last gasp, as shown in young dogs (19) and neonates of rhesus monkeys and guinea pigs (12). Discomfort might be experienced until actual brain damage occurs. Resistance to anoxia shows

substantial inter-individual variation. Upon exposure of 195 rabbit newborns to gaseous nitrogen at 35 °C, individual time to last gasp varied between 7 to 39 minutes, with a mean of 16 minutes (12). For 5 dog newborns exposed to gaseous nitrogen, values of 16 to 40 minutes were found for time to last gasp at unmentioned temperature, with an average of 26 minutes (19). In general, survival time of mammals in the absence of oxygen is a function of species, age, environmental temperature and the mechanism, which causes lack of oxygen (12, 13-15, 17-31, 33).

Unlike adults, fetuses and neonates meet energy requirements during lack of oxygen with anaerobic metabolism (12). Their increased resistance is further suggested to be related to substantially lower cerebral oxygen requirements compared to the adult brain, as shown for a.o. mice, rats, cats and dogs (12, 22, 24, 25). A third protective mechanism is that of redistribution of blood flows in favour of brains, heart and adrenals (21, 23, 28, 29). In general, resistance to anoxia or survival time increases with decreasing gestational age. Because fetuses survive anoxia longer than adults, they may also longer be capable of experiencing discomfort. Therefore also the capability of experiencing pain has to be taken into account.

In conclusion, although anoxia will develop in the fetus after the umbilical cord has been cut, it will not directly result in lowered brain function as it would in adult animals; the fetal calf can be expected to have normal brain function at the time of heart puncture.

Pain and Nociception in the Mammal Fetus

Whether or not a mammal fetus or neonate can experience pain is an issue that only recently gained attention of the scientific and medical community. About ten years ago, it was still thought that human babies are less sensitive to pain; as a result surgery was performed on premature and full-term babies without anaesthesia (34, 35). Recently, different scales of

quantifying pain in human newborns at term (36-38) and pre-term (39, 40) have been developed. When discussing this issue, one should distinguish between pain and nociception. *Pain* refers to a (highly disturbing) sensation, thus requiring some level of consciousness; *nociception* refers to the activity of neural pathways involved in the transmission of noxious (potentially or actually tissue damaging) stimuli. Noxious stimuli do not necessarily lead to pain sensation, but may elicit only reflexes (e.g. rapid withdrawal of affected limb). In deep anaesthesia, both the ascending pathways and nociceptive reflexes are suppressed. For pain to be experienced, the cerebral cortex and subcortical structures have to be functional (41).

The difficulties in describing pain are reflected by the multitude of definitions that has been developed to try to describe this phenomenon (42-51). As animals - and neonates and fetuses of any species - cannot tell an observing human that they are in physical pain, one should base a judgment on what is considered to be reasonable from the available evidence. By consensus, it is agreed that animals can feel pain post-natally (primarily mammals and other vertebrates).

Concerning mammal fetuses, an important question is that of fetal development: at which stage of neural development is a fetus able to experience pain or discomfort? The term discomfort encompasses any pain, distress, stress, discomfort, anxiety and / or fear - thus being the antithesis of comfort or well-being. The newborns of different species are of different maturity at birth. The newborns of rats, rabbits, mice and humans are less well developed than those of guinea pigs, sheep, bovines, equines and pigs (12, 33). By consensus, the human fetus is considered to be able to experience pain as from 24 weeks post-conception (development of thalamo-cortical connections) (35, 38, 52-54), and to have the ability to suffer as from 11 weeks post-conception (35). Suffering results from pain or distress being of sufficient intensity or duration, or both (50). Sheep fetuses are suggested to be capable of perceiving sensory stimuli at 15½ weeks of gestation (term = 20 weeks) (33). Sheep, equine, porcine and bovine fetuses are suggested to be able to feel pain “from the time at which the

neural tube develops into a functional brain" (> 30% gestation) (55). Human, rat and cat fetuses and neonates seem more susceptible to pain than adults, due to immaturity of the brain stem mechanisms which dampen part of nociceptive transmissions in the spine. This mechanism starts to develop in humans gradually not sooner than 33 weeks of gestation; in rats, after day 7 post-partum (35, 52, 56-61). Hence it is suggested that both noxious stimuli and even touch are regarded as nociceptive before this mechanism becomes functional (35, 52, 58). Thus a fetus might even experience some degree of pain or discomfort upon being touched only.

The pain threshold increases with increasing age. In the absence of a functional cortex, lower brain structures may function in a more complex manner than in its presence, as suggested for humans (the thalamus and amygdala in particular may be involved in supporting some basic form of consciousness) (35, 62, 63). It is stated that "the fetus could be aware and have consciousness once lower structures [than the cortex] in the brain are formed. The degree of consciousness is unknown and as yet unknowable" (35). Challenging the cortex of being the sole site of consciousness is a matter of different opinions amongst scientists (35, 54). It is worthwhile to note that it is not only the sense of pain that becomes active at a certain stage of development in a mammal fetus: other recently discovered features of developing mammals are e.g. their ability to learn (64), and to see or respond to visual stimulation (human as from 26 weeks gestational age; sheep as from 16½ weeks of gestation; cats properly as from 26 days post-partum average) (32, 35, 64-66).

In conclusion, since it is likely that the bovine fetus is alive, it is to be expected that it will experience pain and/or suffering at the moment of heart puncture for blood collection and possibly for a period after that, until it actually dies.

Scientific problems of FBS in animal cell culture

There are several technical and scientific problems related to the application of FBS in cell culture. Its price and availability fluctuate due to variations in mondial livestock numbers, importation regulations, beef and dairy prices, livestock feed costs and weather conditions (7, 10, 67).

Batch-to-batch variations (68-70) make it necessary to pre-test every batch before purchase. The presence of many different growth and growth inhibition factors may lead to overgrowth of e.g. fibroblasts in mixed cultures. From a scientific point of view it may be questioned what the effects are of the absolute molecular composition of FBS relative to the serum of the species, gender and developmental stage the cultured cells are derived from (70). Proper cell growth does not necessarily coincide with proper cellular function (3). Fetal bovine serum can interfere with genotypic and phenotypic cell stability (71, 72), and can influence experimental outcome (5, 6, 71, 73-77). Serum can suppress cell spreading, attachment (78) and embryonal tissue differentiation (76). Finally, serum can be contaminated with viruses, bacteria, mycoplasmas, yeast, fungi, immunoglobulins, endotoxins, and possibly prions (11, 67, 72, 79-85). These undesired substances can affect scientific experiments and bulk production of proteins. Many substances present in FBS have not yet been identified (67, 86, 87) and of many substances, which have been identified, the function on the cultured cells is unclear (79).

Discussion

From the questionnaires, conflicting data were obtained with regard to the time of death of the donor fetus. Contrary to the statement of the fetal heart functioning and the fetus being alive during puncture (2 harvesters, 1 scientists, 1 supplier) are statements of entities that claim the

heart does not function during puncture (1 harvester, 1 ministry department), the fetus being dead (1 ministry department, 3 suppliers). One harvester claims fetal anoxia results in a state of cerebral deterioration at the moment of bleeding comparable to that of adult bovines after stunning during slaughter. In the light of these contradictions it is worth mentioning the following. First, the amount of blood obtained by cardiac puncture from a non-functional heart is minimal: only the amount in the heart itself can be harvested (1 supplier, 1 scientist). "Effective electrical stunning causes cardiac arrest and so electrical stunning is not suitable for the harvesting for blood from the heart." (81). The average volumes harvested at 3, 6 and 9 months of gestation indicate circulatory functionality. Secondly, blood coagulates immediately upon death (1 supplier). Thirdly, upon death the Na^+ / K^+ and Ca^{2+} electrochemical equilibria over cell membranes can no longer be maintained, as this requires energy. As a result, Na^+ and Ca^{2+} will diffuse out of tissue fluids into the cells, and K^+ out of cells into tissue fluids (32). The amounts of Na^+, K^+, and Ca^{2+} present in analysed batches of FBS (68, 69) show normal physiological values, be it that K^+-levels are about twofold higher due to the rough means (vacuum) of blood sampling (Duncker, *pers. com*., Erasmus University Rotterdam, Faculty of Medicine, Dept. of Experimental Cardiology, August 9th, 2000). Finally, there are companies who do state that fetal blood harvest through cardiac puncture represents an ethical problem (3 suppliers). From the above, we may conclude that the fetus is alive at the time of cardiac puncture.

Fetal blood collection

Humane treatment implies that slaughterhouse staff, scientists and others involved in the bleeding procedure should protect the fetus from potentially painful stimuli, i.e. guarantee proper pain relief (88, 89). It is immoral to inflict preventable discomfort without good reason: unjustly treating the fetus as insentient has much more negative impact than unjustly

treating it as sentient (90). Since it is very likely that the fetus is alive at the time of blood collection, the degree of neural development should be considered. Incomplete development of the nervous system may result in a more profound experiencing of pain or discomfort in a fetus. Exsanguination and cardiac puncture (penetrating skin, internal and external intercostal muscles, costal pleura, heart muscle, and heart pleura) are both graded as severe discomfort in unanaesthesised post-natal bovines (2). From this, we have to conclude that the current practice of blood collection from fetal bovines causes suffering to these animals.

It has been mentioned that anoxia at the time of blood collection acts as anaesthesia. But, as fetuses survive anoxia longer than adults, they may also experience discomfort longer. It may strongly be questioned to what extent the effects of anoxia could be regarded as proper fetal anaesthesia. A 35-minute arrest of brain circulation in 8-11 day old dogs did not result in loss of flexion reflex until dogs were 45 days of age (19). Reflex responses to cutaneous stimulation in anoxic newborns failed “shortly” before the last gasp (12). These data suggest anoxia did not result in deep anaesthesia. Eventually brain damage occurs and the fetus will die. If a bovine fetus is brain dead at the moment of bleeding, it will not experience discomfort. The question *when* brain damage starts to occur in bovine fetuses at given gestational age as a result of anoxia could not be answered through the literature. Whether or not a fetus experiences discomfort from lack of oxygen in itself is unknown.

Considering the large variation between individuals of the same species in survival time to anoxia, applying anaesthesia before bleeding would be appropriate. But, mammal fetuses and neonates have a poor capacity to metabolise a variety of drugs. For example, 1-day old mice die of doses of hexobarbitone of which adult mice only sleep for less than 5 minutes (91). The method of choice for anaesthesia of bovine fetuses used for FBS harvest need not only to comply with the above mentioned, but also with the aim of the procedure (serum harvest). Close *et al*. state the fetuses of large mammals (like horses and bovines) could be euthanised

by any means, which is considered acceptable for an adult (55). The use of different chemicals like barbiturates may increase batch-to-batch variations and taint the serum itself. A mechanical method, like a captive bolt, seems more appropriate. A bolt will induce brain death if appropriately used.

Reducing and replacing FBS in animal cell culture

Since FBS is undefined, its application in culture media may alter the outcome of scientific experiments involving cell cultures and make it difficult to compare similar experiments performed with different batches of serum (6, 76). Hence, FBS may interfere with the advancement of biological science (92, 93). FBS should be replaced or its use reduced in cell culture both on scientific and moral grounds. As long as fetuses may be expected to experience discomfort from the methods employed in FBS harvest it is a morally problematic product. Even if a fetus was stunned prior to blood harvest the scientific problems discussed above remain. Hence it is worthwhile to consider methods of reducing or replacing FBS in cell culture. Most chemically defined (= synthetic) media are cell-type specific (7, 78, 94). Most cell types would have been cultured successfully in synthetic media, except for endothelial cells (72).

Another way of replacing FBS in media is using another type of animal derived serum (7, 86). However, using animal sera inherently concerns animal welfare, apart from the question if cells will grow well with another serum type. Fetal horse and fetal pig serum are harvested by cardiac puncture as well and hence are no replacement alternatives to FBS. Concerns also exist related to newborn calf serum production, e.g. suffering due to transport.

When a certain cultured cell type does not allow for replacement of FBS, applying reduced-serum media can decrease its use. The volume of FBS in culture media varies between 5 and 20% (93). Usually, growth factors should be added to such media (7). Getting cells

accustomed to a different culture medium composition often requires an adaptation or weaning period; weaned DG44 cells showed improved cell growth in serum-free medium, relative to unweaned DG44 cells (95). Finally, several institutions and companies offer the service of developing serum-free media upon request.

Conclusion

Fetal bovine serum is both a scientifically and a morally problematic product. Its application in cell culture experiments represents a scientific problem as FBS is undefined and may interfere with the outcome of experiments. Harvesting bovine fetal blood by cardiac or umbilical cord puncture without the refinement of pain relief, such as stunning with a captive bolt, can be considered immoral. This has consequences for cell culture applications, which currently rely on FBS as a medium component. The thought that cell culture techniques requiring FBS are a replacement to the use of animals is a misconception. If refinement of fetal blood harvesting methods can be endorsed, several scientific and technical problems inherent to the biological origin of sera remain. It should therefore be questioned why animals are used at all for such product. On moral and scientific grounds the most promising alternative to FBS is the use of defined media.

Recommendations

- A discussion should be initiated on ethical and scientific aspects of the use of FBS.

- The use of FBS for specific human or animal interests should be balanced against the discomfort of the used animals.
- The development and subsequent use of synthetic media should be encouraged

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References

01. Weihe, W.H. (1985). Use and misuse of an imprecise concept: alternative methods in animal experiments. *Laboratory Animals* **19**, 19-26.

02. van Zutphen, L.F.M., Baumans, V. & Beynen, A.C. (ed.). (2001). *Principles of Laboratory Animal Science*, revised edn, 416 pp. Amsterdam, NL: Elsevier Science Publishers B.V.

03. Gardner, D.K. (1998). Development of serum-free media for the culture and transfer of human blastocysts. *Human Reproduction* **14** (S4), 218-225.

04. Balls, M., Goldberg, A.M., Fentem, J.H., Broadhead, C.L., Burch, R.L., Festing, M.F.W., Frazier, J.M., Hendriksen, C.F.M., Jennings, M., van der Kamp, M.D.O., Morton, D.B., Rowan, A.N., Russell, C., Russell, W.M.S., Spielmann, H., Stephens, M.L., Stokes, W.S., Straughan, D.W., Yager, J.D., Zurlo, J. & van Zutphen, B.F.M. (1995). The three Rs: the way forward. *Alternatives To Laboratory Animals* **23**, 838-866.

05. Kohlpoth, M. & Rusche, B. (1997). Kultivierung einer permanenten Fischzellinie in serumfreien Medien: Spezielle Erfahrungen mit einem Zytotoxizitätstest für Abwasserproben. *Alternatieven zu Tierexperimenten* **14**, 16-20.

06. Kohlpoth, M. (1998). Kultivierung von Zellinien in serumfreien Medien: Generelle Vorteile und spezielle Erfahrungen in einem Zytotoxizitätstest mit Fischzellen. In: *Ersatz - und Ergänzungsmethoden zu Tierversuchen. Forschung ohne Tierverusche 1997* (eds. H. Schöffl, H. Spielmann, H.A. Tritthart), pp. 15-23. Wien, Austria: Springer Wien.

07. Jayme, D.W., Epstein, D.A. & Conrad D.R. (1988). Fetal bovine serum alternatives. *Nature* **334**, 347-348.

08. Anonymous (1993). “Total grausames geschehen”. *Der Spiegel* **4**, 190-193.

09. Hodgson, J. (1995). To treat or not to treat: that is the question for serum. *Bio/Technology* **13**, 333-343.

10. Strouken, H.P.M. (1995). Koeien als leverancier bloedplasma. *Oogst* 12 mei 1995: 44.

11. Hodgson, J. (1993). Fetal bovine serum revisited. *Bio/Technology* **11**, 49-55.

12. Dawes, G.S. (1968). *Foetal and Neonatal Physiology*, 247 pp. Chicago, US: Year Book Medical Publishers.

13. Rosenfeld, M. & Snyder, F.F. (1938). Stages of development of respiratory regulation and the changes occuring at birth. *American Journal of Physiology* **121**, 242-249.

14. Glass, H.G., Snyder, F.F. & Webster, E. (1944). The rate of decline in resistance to anoxia of rabbits, dogs, and guinea pigs from the onset of viability to adult life. *American Journal of Physiology* **140**, 609-615.

15. Avery, R.C. & Johlin, J.M. (1932). Relative susceptibility of adult and young mice to asphyxation. *Proc Soc Exp Biol and Med* **29**, 1184-1186.

16. Reiß, M. (1931). Das Verhalten des Stoffwechsels bei der Erstickung neugeborener Ratten und Mäuse. *Zeitschrift für d ges exper Med* **79**, 345-359.

17. Reiß, M. & Haurowitz, F. (1929). Über das verhalten junger und alter Tiere bei Erstickung. *Klinische Wochenschrift* **8**, 743-744.

18. Woolley, S.C. & Gentle, M.J. (1988). Physiological and behavioural responses of the domestic hen to hypoxia. *Research in Veterinary Science* **45**, 377-382.

19. Kabat, H. (1940). The greater resistance of very young animals to arrest of the brain circulation. *American Journal of Physiology* **130**, 588-599.

20. Richardson, B.S. (1992). Metabolism of the fetal brain: biological and pathological development. In: *The Fetal and Neonatal Brain Stem* (ed. M.A. Hanson), pp. 87-105. Cambridge, United Kingdom: Cambridge University Press.

21. Ashwal, S.P., Dale, P.S. & Longo, L.D. (1984). Regional cerebral blood flow: studies in the fetal lamb during hypoxia, hypercapnia, acidosis and hypotension. *Pediatric Research* **18**, 1309-1316.

22. Gregoire, N.M., Gjedde, A., Plum, F. & Duffy, T.E. (1978). Cerebral blood flow and cerebral metabolic rates for oxygen, glucose, and ketone bodies in newborn dogs. *Journal of Neurochemistry* **30**, 63-69.

23. Jensen, A. (1996). The brain of the asphyxiated fetus - basic research. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **65**, 19-24.

24. Potter, V.R., Schneider, W.C. & Liebl, G.J. (1945). Enzyme changes during growth and differentiation in the tissues of the rat. *Cancer Research* **5**, 21-24.

25. Zarchin, N. & Mayevsky, A. (1981). The effects of age on the metabolic and electrical repsonses to decapitation in the awake and the anaesthesized rat brain. *Mechanisms of Ageing and Development* **16**, 285-294.

26. Lagercrantz, H. & Slotkin, H. (1986).The "stress" of being born. *Scientific American* **254**, 92-102.

27. Carr, B.R. (1993). Fertilization, implantation and endocrinology of pregnancy. In: *Textbook of Endocrine Physiology* (ed. J.E. Griffin, S.R. Ojeda), 2nd edition, pp. 189-209. Oxford, UK: Oxford University Press.

28. Hanson, M. (1992). The control of the fetal circulation. In: *The Fetal and Neonatal Brain Stem* (ed. M.A. Hanson), pp. 59-85. Cambridge, United Kingdom: Cambridge University Press.

29. Jansen, A.H., Belik, J., Ioffe, S. & Chernick, V. (1989). Control of organ blood flow in fetal sheep during normoxia and hypoxia. *American Journal of Physiology* **257**, 1132-1139.

30. Blackmore, D.K. (1984). Differences in behaviour between sheep and cattle during slaughter. *Research in Veterinary Science* **37**, 223-226.

31. Derbyshire, S.W. & Bennet, P. (1994). Fetal stress responses. *Lancet* **344**, 615.

32. Gluckman, P.D., Williams, C.E. & Gunn, A.J. (1992). Brain stem and cerebral function in the fetus: its assessment and the impact of asphyxia. In: *The Fetal and Neonatal Brain Stem* (ed. M.A. Hanson), pp. 185-210. Cambridge, United Kingdom: Cambridge University Press.

33. Reilly, J.S. (ed.) (1993). *Euthanasia of animals used for scientific purposes*, 73 pp. Glen Osmond, Australia: Australian and New Zealand Council of the Care of Animals in Research and Teaching.

34. de Boo, M. (1997). Pijn is nergens goed voor. *Hypothese* **12**, 4-5.

35. The Rt Hon Lord Rawlinson of Ewell, Alton, D., Ames, D., Booth, H., Colchester, C., The Baroness Cox, Farmer, A., Francoise, C., Gormally, F., Graffius, C., Hendron, J., The Rt Hon the Lord Jacobovirs, Langdon-Stokes, M., The Baroness Masham of Ilton, McLean, J., Moore, P., Nolan, M., Countess Quintavalle, Scarisbrick, J., Taylor, R., White, M., Wyatt, J. & Bartley, J. (1996). *Report of the Commission of Inquiry into Fetal Sentience*, 24pp. London, United Kingdom: CARE Trust.

36. Gradin, M. (2000). Need for a reliable pain evaluation scale in the newborn in Sweden. *Acta Anaesthesiol Scand* **44**, 552-554.

37. van Dijk, M., de Boer, J.B., Koot, H.M., Tibboel, D., Passchier, J. & Duivenvoorden, H.J. (2000). The reliability and validity of the COMFORT scale as a postoperative pain instrument in 0 to 3-year-old infants. *Pain* **84**, 367-377.

38. Van Lingen, R.A. (2000). *Pain assessment and Analgesia in the Newborn: an integrated approach*, 177 pp. Rotterdam, The Netherlands: Erasmus University.

39. Ballantyne, M., Stevens, B., McAllister, M., Dionne, K. & Jack, A. (1999). Validation of the premature infant pain profile in the clinical setting. *Clin J Pain* **15**, 297-303.

40. Scott, C.S., Riggs, K.W., Ling, E.W., Fitzgerald, C.E., Hill, M.L., Grunau, R.V., Solimano, A. & Craig, K.D. (1999). Morphine pharmacokinetics and pain assessment in premature newborns. *J Pediatr* **135**, 423-429.

41. Beaver, B.V., Reed, W., Leary, S., McKiernan, B., Bain, F., Schultz, R., Taylor Bennet, B., Pascoe, P., Shull, E., Cork, L.C., Francis-Floyd, R., Amass, K.D., Johnson, R., Schmidt, R.H., Underwood, W., Thornton, G.W. & Kohn, B. (2000). 2000 Report of the AVMA panel on euthanasia. *Journal of the American Veterinary Medical Association* **218**, 669-696.

42. Merskey, H., Albe-Fessard, D.G., Bonica, J.J., Carmon, A., Dubner, R., Kerr, F.W.L., Lindblom, U., Mumford, J.M., Nathan, P.W., Noordenbos, W., Pagni, C.A., Renaer, M.J., Sternbach, R.A. & Sunderland, S. (1979). Pain Terms: a list with definitions and notes on usage: recommended by the IASP Subcommittee on Taxonomy. *Pain* **6**, 249-252.

43. Jessell, T.M. & Kelly, D.D. (1991). Pain and analgesia. In: *Principles of Neural Science* (eds. Kandel E.R., Schwartz, J.H. & Jessell, T.M.)., 3rd edition, 1135 pp. London, United Kingdom: Prentice-Hall International Inc.

44. Close, B., Banister, K., Baumans, V., Bernoth, E.M., Bromage, N., Bunyan, J., Erhardt, W., Flecknell, P., Gregory, N., Hackbarth, H., Morton, D. & Warwick, C. (1996). Recommendations for euthanasia of experimental animals: Part 1. *Laboratory Animals* **30**, 293-316.

45. Kitchell, R.L. (1987). Problems in defining pain and peripheral mechanisms of pain. *Journal of the American Veterinary Medical Association* **191**, 1195-1199.

46. Anonymous (1995). *Code of Recommendations and Minimum Standards for the care and Use of Animals for Scientific Purposes*, 63pp. Wellington, New Zealand: Ministry of Agriculture and Fisheries, Animal Welfare Advisory Committee.

47. Anonymous (1990). *Australian Code of Practice for the care and use of animals for scientific purposes*, 5th edition, 75 pp. Canberra, Australia: Australian Government Publishing Service.

48. Flecknell, P.A. (1984). The relief of pain in laboratory animals. *Laboratory Animals* **18**, 147-160.

49. Wallace, J., Stanford, J., Smith, M.W. & Spencer, K.V. (1990). The assessment and control of the severity of scientific procedures on laboratory animals. *Laboratory Animals* **24**, 97-130.

50. Baumans, V., Brain, P.F., Brugére, H., Clausing, P., Jeneskog, P. & Perreta, G. (1994). Pain and distres in laboratory rodents and lagomorphs. *Laboratory Animals* **28**, 97-112.

51. de Grazia, D. (2000). Philosophical and ethical issues concerning pain. In: *Pain management and Humane Endpoints*. Web site http://altweb.jhsph.edu/science/meetings/pain/program.htm. Baltimore, United States of America: Altweb, Center for Alternatives to Animal Testing.

52. Fitzgerald, M. (1994). Neurobiology of fetal and neonatal pain. In: *Textbook of Pain* (eds. P.D. Wall & R. Melzack), 3rd edn, pp. 153-163. London, United Kingdom: Churchill Livingstone.

53. Vanhatalo, S. & van Nieuwenhuizen, O. (2000). Fetal pain? *Brain Dev* **22**, 145-50.

54. Wise, J. (1997). Fetuses cannot feel pain before 26 weeks. *Britisch Medical Journal* **315**, 1111-1116.

55. Close, B., Banister, K., Baumans, V., Bernoth, E.M., Bromage, N., Bunyan, J., Erhardt, W., Flecknell, P., Gregory, N., Hackbarth, H., Morton, D. & Warwick, C. (1997). Recommendations for euthanasia of experimental animals: Part 2. *Laboratory Animals* **31**, 1-32.

56. Terman, G.W., Shavit, Y., Lewis, J.W., Cannon, J.T. & Liebeskind, J.C. (1984). Intrinsic mechanisms of pain inhibition: activation by stress. *Science* **226**, 1270-1277.

57. Paterson, D.J., Robson, L.E. & Kosterlitz, H.W. (1983). Classification of opioid receptors. *British Medical Bulletin* **39**, 31-36.

58. Fitzgerald, M. (1992). The development of descending brain stem control of spinal cord sensory processing. In: *The Fetal and Neonatal Brain Stem* (ed. M.A. Hanson), pp. 127-135. Cambridge, United Kingdom: Cambridge University Press.

59. Lagercrantz, H. & Forssberg, H. (1991). Hjärmans funktionella utveckling hos fostret och spädbarnet. *Nordisk Medicin* **106** (10), 264-268.

60. del Fiacco, M., Dessi, M.L. & Levanti, M.C. (1984). Topographical localization of substance P in the human post mortem brain stem: an immunohistochemical study in the newborn and adult tissue. *Neuroscience* **12**, 591-611.

61. Lagercrantz, H. & Srinivasan, M. (1992). Development and function of neurotransmitter / modulator systems in the brain stem. In: *The Fetal and Neonatal Brain Stem* (ed. M.A. Hanson), pp. 1-19. Cambridge, United Kingdom: Cambridge University Press.

62. McCullagh, P. (1997). Do fetuses feel pain? Can fetal suffering be excluded beyond reasonable doubt? *British Medical Journal* **314**, 302.

63. Saunders, PJ. (1997). Do fetuses feel pain? We should give them the benefit of the doubt. *British Medical Journal* **314**, 302.

64. Bernstein *et al.* according to Hepper, P.G. (1992). Fetal psychology: an embryonic science. In: *Fetal Behaviour* (ed. J.G. Nijhuis), pp. 129-156. Oxford, United Kingdom: Oxford Medical Publications.

65. Gagnon, R. (1992). Fetal behaviour in relation to stimulation. In: *Fetal Behaviour* (ed. J.G. Nijhuis), pp. 209-226. Oxford, United Kingdom: Oxford Medical Publications.

66. Rowe, M. (1992). Coding of tactile information in the developing nervous sytem. In: *The Fetal and Neonatal Brain Stem* (ed. M.A. Hanson), pp. 137-160. Cambridge, United Kingdom: Cambridge University Press.

67. Johnson, R.W. (1995). Fetal bovine serum: optimizing cell culture media (reprinted from *IVD Technology*, February 1995), 3 pp. Verviers, B: Boehringer Ingelheim Bioproducts.

68. Honn, K.V., Singley, J.A. & Chavin, W. (1975). Fetal bovine serum: a multivariate standard. *Proceedings of the Society for Experimental Biology and Medicine* **149**, 344-347.

69. Price, J.P. & Gregory, E.A. (1980). *Relationship between in vitro growth promotion and biophysical and biochemical properties of the serum supplement*. Version of submitment, 18 pp. Obtained from: Berlin, Germany: Biochrom Beteiligungs GmbH & Co., Produktionsgesellschaft.

70. Knepper, P.A., Mayanil, C.S., Goossens, W., McLone, D.G. & Hayes, E. (1998). The presence of transcription factors in fetal bovine sera. *In Vitro Cell. Dev. Biol - Animal* **34**, 170-173.

71. Newman Jr., K. (2000) *Serum-Free Systems: Essential for critical work and contemporary routine tissue culture*. Web site http://www.celox.com. St. Paul, United States of America: Celox Laboratories, Inc.

72. DeFrancesco, L. (1998). Serum-free cell culture. *The Scientist* **12**, 19-21.

73. Bruno, J.G., Herman, T.S., Cano, V.L., Stribling, L. & Kiel, J.L. (1999). Selective cytotoxicity of 3-amino-l-tyrosine correlates with peroxidase activity. *In Vitro Cell. Dev. Biol - Animal* **35**, 376-382.

74. Yang, F., Abdel-Malek, Z. & Boissy, R.E. (1999). Effects of commonly used mitogens on the cytotoxicity of 4-tertiary butylphenol to human melanocyte. *In Vitro Cell. Dev. Biol - Animal* **35**, 566-570.

75. Peehl, D.M., Sellers, R.G. & Wong, S.T. (1998). Defined medium for normal adult human prostatic stromal cells. *In Vitro Cell. Dev. Biol - Animal* **34**, 555-561.

76. Bulic-Jakuš, F., Vlahovic, M., Juric-Lekic, G., Crnek-Kunstelj, V. & Šerman, D. (1999). Gastrulating rat embryo in a serum-free culture model: changes of development caused by teratogen 5-azacytidine. *Alternatives To Laboratory Animals* **27**, 925-933.

77. Sobrero, A.F. & Bertino, J.R. (1986). Endogenous thymidine and hypoxanthine are a source of error in evaluating methotrexate cytotoxicity by clonogenic assays using undialyzed fetal bovine serum. *International Journal of Cell Cloning* **4**, 51-62.

78. Katayama, H., Kino, J., Itami, S., Tsutsui, M., Koizumi, H., Halprin, K.M. & Adachi, K. (1986). A cell-spreading inhibitor exists in serum and in epidermal basal cells. *Journal of Investigative Dermatology* **86**, 694-699.

79. Sumter, R.D. (1993). *Literatuur onderzoek naar het potentieel gebruik van foetaal kalfserum in industrie en onderzoeksinstituten*, 36 pp. Utrecht, The Netherlands: Utrecht University.

80. Erickson, G.A., Bolin, S.R. & Landgraf, J.E. (1991). Viral contamination of fetal bovine serum used for tissue culture: risks and concerns. *Develop Biol Stand* **75**, 173-175.

81. Anonymous (1996). *Guidelines for the welfare of livestock from which blood is harvested for commercial and research purposes*, 15 pp. Wellington, New Zealand: Ministry of Agriculture, Animal Welfare Advisory Committee.

82. Kajander, E.O., Kuronen, I. & Ciftcioglu, N. (1996). Fatal (fetal) bovine serum: discovery of Nanobacteria. *Mol. Biol. Cell, Suppl*., **7**, 517a.

83. Ciftcioglu, N., Hiltunen, E., Akerman, K. & Kajander, E.O. (1996). Apoptotic effect of Nanobacteria on cultured mammalian cells. *Mol. Biol. Cell, Suppl*., **7**, 517a.

84. Ciftcioglu N., Kuronen I., Akerman K., Hiltunen E., Laukkanen J. & Kajander E.O. (1997). A New Potential Threat in Antigen and Antibody Products: Nanobacteria. In: *Vaccines 97* (eds. Brown & al.), pp. New York, US: Cold Spring Harbor Laboratory Press.

85. Angulo, A.F., Reijgers, R., Brugman, J., Kroesen, I., Hekkens, F.E.N., Carle, P., Bové, J.M., Tully, J.G., Hill, A.C., Schouls, L.M., Schot, C.S., Roholl, P.J.M. & PolakVogelzang, A.A. (2000). *Acholeplasma vituli* sp. nov., from bovine serum and cell cultures. *International Journal of Systematic and Evolutionary Microbiology* **50**, 1125-1131.

86. Anderson, I. (1980). Fetal calf serum drought hits cell culture laboratories. *Nature* **285**, 63.

87. YuanLong, P., Bender, P.K., Akers, R.M. & Webb, K.E.J. (1998). One or more serum factors promote peptide utilization in cultured animal cells. *Journal of Nutrition* **128**, 744-750.

88. Russell, W.M.S. & Burch, R.L. (1959). *The principles of humane experimental technique*. Web site http://altweb.jhsph.edu/science/pubs/humane_exp/het_toc.htm. Baltimore, United States of America: Altweb, Center for Alternatives to Animal Testing.

89. Russell, W.M.S. (1999). The progress of humane experimental technique. *Alternatives To Laboratory Animals* **27**, 915-922.

90. Stafleu, F.R., Rivas, E., Rivas, T., Vorstenbosch, J., Heeger, F.J. & Beynen, A.C. (1992). The use of analogous reasoning for assessing discomfort in laboratory animals. *Animal Welfare* **1**, 77-84.

91. Jondorf, W.R., Maickel, R.P. & Brodie, B.B. (1958). Inability of newborn mice and guinea pigs to metabolise drugs. *Biochem Pharmacol* **1**, 352-354.

92. de Jager, C. (1991). De wetenschappelijke methoden. *Skeptische Notities* **7**, 8-27.

93. Arnaud, P., Paddock, G.V. & Dolan, J.W. (2001). A replacement for fetal bovine serum: recombinant fetuin? *Alternatives Today* Spring 2001, 1,8.

94. Rizzino, A., Rizzino, H. & Sato, G. (1979). Defined media and the determination of nutritional and hormonal requirements of mammalian cells in culture. *Nutrition Reviews* **37** (12), 369-376.

95. Kim, E.J., Kim, N.S. & Lee, G.M. (1999). Development of a serum-free medium for dihydrofolate reductase-deficient chinese hamster ovary cells (DG44) using a statistical design: beneficial effect of weaning of cells. *In Vitro Cell. Dev. Biol - Animal* **35**, 178-182.

Diagram summarizing the fetal blood collection process