ORIGINAL

ABSTRACT OF INVENTION:

Title: Extractegg-a novel alternative to Fetal bovine serum (FBS) in animal cell culture

Fetal bovine serum (FBS) is a common component of all animal cell culture media. FBS is harvested from fetuses of pregnant cow during slaughter. Being live at the time of blood collection, these fetuses are exposed to severe pain and stress. Scientific and ethical concerns are existing regarding the use of FCS in *in vitro* culture. Across the world several studies are on progress to find a suitable media which can maintain the immortality of stem cell and which can provide a suitable environment for the in vitro produced embryos. Therefore the present study was conducted to exploit the potential of Egg Yolk Extract (EYE) as a possible alternative to FBS in various animal cell cultures and in vitro embryo production. ' Extrategg' was prepared from fresh unfertilized chiken egg after proper processing. They were used at a concentration of 2% in all the cell culture media used Vs 10% FBS. Caprine / Bovine fetal stem cells and fibroblast cells had normal growth and multiplication in the Egg Extract supplemented media which were later characterized with stem cell specific markers. The new media could maintain the fertilization rate in caprine and bovine *in vitro* embryo production (both invitro fertilization and parthenogenesis)

11. DATE AND SIGNATURE:

Dated....27th.....of ...Sept.....2010

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To,
The Controller of Patents,
The Patent Office, New Delhi

5. CLAIMS:

I / we claim

1. Caprine fetal somatic cell culture- Caprine whole body fetal cells were isolated using standard protocol and cultured with 2% 'Extractegg' which was comparable to 10% FBS.

2. Caprine fetal stem cell culture – Caprine fetal stem cells were isolated using standeard protocol and cultured with 4% 'Extractegg' which was comparable to 20% FBS.

3. Caprine fetal fibroblast cell culture- Caprine fetal fibroblast monolayer was isolated and cultured using standard protocol with 2% 'Extractegg' which was comparable to10% FBS

4. Caprine / bubaline in vitro embryo development (Both IVF and parthenogenesis),- FBS was replaced with Extractegg successfully in *in vitro* oocyte maturation and embryo culture at the rate of 2% Vs 10% in Caprine and bubaline embryo development procedure.

5. The technique describes the method of preparation of 'Extractegg' a novel media constituent form chicken egg which can replace FBS in cell culture

6. Extractegg could successfully replace FBS in Caprine / bubaline in vitro embryo development procedure

7. Extractegg could successfully replace FBS in caprine fetal stem cell culture, caprine fetal fibroblast culture and caprine somatic cell culture at a very low concentration comparatively.

8. The fetal cells culture in 'Extractegg ' were also found positive for the major stem cells specific markers Oct4, Nanog and Sox2

9. "Extractegg" can successfully replace FBS

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3. PREAMBLE TO THE DESCRIPTION

COMPLETE

3.1 PRIOR ART

Cell and tissue culture, i.e. the propagation and cultivation of cells *in vitro* has become an indispensable tool in biomedical sciences. Recently the use of cell culture is increasing day by day to decrease use of animal in experiment to stop cruelty to them. A complete and proper media for culture of all the type of cells of body is lacking and new *in vitro* alternatives are constantly being developed. Areas like cell culture vaccine technology, stem cell technology, assisted reproductive technology cannot survive without the support of cell culture techniques. The routine cell and tissue culture demands the use of animal derived products, mainly fetal bovine serum (FBS), as a culture medium constituent. FBS contains all the fundamental hormones and several growth factors required for cell division, growth and survival. The amount of FBS produced for the world market is estimated at approximately 5 lakh litres per year. For this more than 10 lakh bovine fetuses have to be harvested, and it is expected that, these numbers will continue to increase annually (Jochems et al., 2002). FBS is manufactured from blood that has been harvested from calves which are removed from a cow's uterus after slaughter of the dam. Usually, the blood is obtained by cardiac puncture without the use of anesthesia. There is increasing evidence that calves may be alive and, if they have breathed in air, may have normal

brain function during the blood collection process. Alternatively, fetal blood may be harvested by means of umbilical vein puncture or puncture of the jugular vein (Jochems et al., 2002). Further it is required to test the FBS for different diseases and only the disease free FBS are used for cell culture technology. The cost of FBS is thereby very high.

The role of serum in cell culture media

The supplementation of basal culture media with animal serum of different origin is essential for cell growth and for the stimulation of proliferation. The sera used most widely are from adult, newborn, or of fetal bovine origin. Whole animal serum as well as fetal bovine serum (FBS), in general, is an extremely complex mixture of a large number of constituents, low and high molecular weight biomolecules with different, physiologically balanced growth-promoting and growth-inhibiting activities. Contemporary knowledge in modern cell biology and biochemistry allowed the identification of the (growth) factors involved in *in vivo* processes, like cell proliferation and tissue repair (e.g. in wound healing), and cell maturation and differentiation (e.g. embryonic maturation, stem cell lineage, epithelial differentiation, etc.). The major functions of serum in culture media are to provide (i) hormonal factors stimulating cell growth and proliferation, and promoting differentiated functions, (ii) transport proteins carrying hormones (e.g. transcortin), minerals and trace elements (e.g. transferrin), and lipids (e.g. lipoproteins), (iii) attachment and spreading factors (i.e. components of the extracellular matrix), and (iv) stabilizing and detoxifying factors, needed to maintain pH or to inhibit proteases either directly, such as a-antitrypsin or α2-macroglobulin, or indirectly, by acting as an unspecific sink for proteases and other (toxic) molecules because of its rich content of growth factors and its low gamma globulin content. FBS has become the standard supplement of cell culture media. In most cases, FBS is used at concentrations of 10-20% (v/v), although this may vary for specific applications (e.g. low serum media). Other advantages in the use of FBS include the following: (i) FBS is a cocktail of most of the factors required for cell proliferation and maintenance (ii) it is thus an almost universal growth supplement effective in most types of human and animal cells. (iii) Using serum supplemented medium therefore reduces the need to spend time and effort developing a specific, optimized medium formulation for each cell type. However, the use of animal serum in cell culture also bears a number of disadvantages (van der Valk et al., 2003): (i) serum is an ill-defined medium supplement, and thus an ambiguous factor in cell culture, (ii)

serum batches display quantitative and qualitative variations in their composition, and thus introduce a significant lot-to-lot variability (iii) serum may contain different amounts of endotoxins, haemoglobin, and other adverse factors, and (iv) serum can be a potential source of microbial contaminants, such as fungi, bacteria, viruses or prions. Thus, serum introduces several unknown variables into the tissue culture system. In addition, serum-supplemented media may be unable to support growth of specific cell types, and in primary cultures of highly differentiated epithelial cells, serum may be unable to prevent the overgrowth of the culture by fibroblasts.

Alternatives to fetal bovine serum in cell and tissue culture

The removal of serum from the cell culture medium and/or the replacement with other complex biological fluids and extracts initiates multifarious variations in the interactive nature of the cell culture system that are introduced in a single step. Nevertheless, methods to reduce the requirements for FBS in culture media as well as alternative animal serum substitutions were described (Jayme et al., 1988). The latter include substitution with newborn or adult calf sera, or the use of sera from other animal species (horse, pig, goat, etc.). Other animal-derived alternatives are tissue extracts (e.g. pituitary extracts, chicken embryo extracts), bovine milk fractions, or bovine colostrum. Most recently, protein fractions from plant extracts, called vegetal serum, were successfully introduced to grow and maintain epithelial cells in culture (Hartung et al., 2003). Additional methods of choice to reduce FBS requirement include (i) the optimization of existing culture media, (ii) the use of reduced serum media, or (iii) the design of serum free, chemically defined media.

Serum-free cell culture

Today, the importance of culturing mammalian cells in medium without serum supplementation is widely recognized (Bjare, 1992). With the identification, cloning, and recombinant production of essential growth factors and nutrients required by different cell types broad selection of chemically defined, serum-free media for continuous cell lines as well as for specific cell types in primary culture has been designed and is available. The advantages and benefits of serum-free media are easily deducible: (i) chemically defined and controlled culture conditions *in vitro*, (ii) reduced variability in qualitative and quantitative culture medium composition, (iii) elimination of a potential source of microbial contamination (iv) advantages in

down-stream processing (i.e. the isolation of cell culture products), and (v) most of all, ethical aspects, i.e. reduction of the worldwide use of FBS and thus the numbers of bovine fetuses used as source of FBS (van der Valk et al., 2003). A single yolk extract factor (EYF-X) obtained from chicken eggs which facilitates the in vitro culture of a variety of cell types including permanent neuronal cell line N2A (Sasse M et al 2000), which they suggest to be investigated in other cells.

3.2 INNOVATION

To the best of our knowledge, we have developed a new cell culture ingredient **'Extractegg'** which can replace the use of FBS in animal cell culture , which have been successfully tested on caprine fetal stem cells, caprine and bovine in vitro embryo development media, caprine and bovine fetal fibroblast and other cell culture etc.

3.3 ADVANTAGE OF THE TECHNOLOGY

1. Slaughter of animals for FBS collection may stopped and thus ethical concerns can be avoided
2. Easy collection and processing procedure which may be done even in low cost laboratory and farms
3. Require smaller quantity for cell growth and proliferation
4. A lot many animal derived factors and variability may be avoided
5. Highly cost effective

3.4 COMMERCIAL VIABILITY

Commercially unfertilized fresh chicken eggs are available which the basic constituent of **'Extractegg'**. As it will be a fresh egg extract in DPBS (Dulbeccos phosphate buffer saline) / NSS (Normal Saline Solution) after separation of large molecular weight proteins and sterilization, it will be very low cost product compared to FBS. Thus 'Extractegg' has got high commercial viability.

4. DESCRIPTON

In this invention a new culture ingredient is described which can replace the use of fetal bovine serum in animal cell culture. The study describes the potential of 'extractegg' to be used in a wide variety of cell cultures including stem cells and in in *vitro* embryo development in various species.

4.1 Preparation of Extractegg media

'Extractegg' media was prepared from fresh unfertilized chicken eggs soon after laying.

4.2 'Extrategg' preparation

The chicken eggs were transferred to laboratory soon after collection. In the lab, the egg shell was swabbed with spirit. The egg shell was then broken and entire egg content was aseptically collected in a glass beaker and was mixed with DPBS / NSS (1:1) and filtered through suction followed by centrifuge at the rate of 3000 rpm for 10 minutes. The supernatant was again subjected to ultra centrifugation at the rate of 12000-16000 rpm for 30 minutes. Again the precipitate was removed and the supernatant was collected. The media was then sterilized using filter 0.45μm and stored at 4^0C till further use.

4.3 Use of 'Extrategg' in different cell culture conditions

Caprine fetal somatic cell culture- Caprine whole body fetal cells were isolated using standard protocol and cultured with 2% 'extractegg' which was comparable to 10% FBS.

Caprine fetal stem cell culture – Caprine fetal stem cells were isolated using standard protocol and cultured with 4% 'extractegg' which was comparable to 20% FBS.

Caprine fetal fibroblast cell culture- Caprine fetal fibroblast monolayer was isolated and cultured using standard protocol with 2% 'extractegg' which was comparable to10% FBS

Caprine / bubaline in vitro embryo development (Both IVF and parthenogenesis),- FBS was replaced with Extract egg successfully in *in vitro* oocyte maturation and embryo

culture at the rate of 2% Vs 10% in Caprine and bubaline embryo development procedure.

4.4 Salient features of the technique

1. The technique describes the method of preparation of 'extractegg' a novel media constituent form chicken egg which can replace FBS in cell culture
2. Extractegg could successfully replace FBS in Caprine / bubaline in vitro embryo development procedure
3. Extractegg could successfully replace FBS in caprine fetal stem cell culture, caprine fetal fibroblast culture and caprine somatic cell cultue at a very low concentration comparatively.
4. The fetal cells culture in 'extractegg ' were also found positive for the major stem cells specific markers Oct4, Nanog and Sox2

4.5 Evaluation of the technique

The protocol describes the formulation of extractegg as a media constituent alternative to FBS which can be used in various types cell culture / embryo cultures.

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