Testing the Milk and Colostrum of Goat as an Alternative for Fetal Bovine Serum in Mammalian Cell Cultures

Grant Proposal

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Author Note

This grant proposal was designed with the support of Dr. Crowthers and Dr. Alatalo who supervised this project, the development of this proposal, and helped advise my progress.

Abstract

Fetal Bovine Serum (FBS) is widely used in culturing cells as it is a very versatile and efficient growth medium. However, FBS is extracted from the blood of a cow fetus and is often very expensive with prices always on the increase. Mainly, the cost of FBS prevents developing areas from conducting research. This project will investigate the effect of using goat's and cow's milk as an alternative for FBS by culturing multiple cell lines using FBS, goat milk and cow milk. The project was initiated to find a cheaper and more ethical alternative for FBS to enable research in environments with lower budgets.

Keywords: Fetal Bovine Serum, bovine milk, goat milk, bovine colostrum, goat colostrum, cell cultures, serum free alternatives.

Testing the Milk of Cow and Goat as an Alternative for Fetal Bovine Serum in Cell Cultures

Fetal Bovine Serum is a widely used growth media for cell cultures but is manufactured unethically and is very expensive. This project will investigate the effect of using cow's and goat's milk as an alternative for FBS. The projects' goal will be achieved by culturing multiple cell lines using FBS, goat milk, and goat colostrum and comparing the cell growth and quality.

The Use of Cell Cultures and The Role of Fetal Bovine Serum (FBS)

Cell cultures are essential in the field of biology as they are used in many forms of research such as understanding the behaviors of wild type cells and diseased cells. Tissue engineering and cellular agriculture are also two fields of study focusing on cell cultures. The process of growing cells requires a growth media that contains the required nutrients to enable the cells to proliferate. Currently, the most prevalent growth media used in the scientific community is Fetal Bovine Serum (FBS), which is derived from the blood of an unborn calf (Lee et al., 2022). Currently, the only legal way to get blood from an unborn calf is from accidental slaughter of pregnant cows which has both has ethical and sustainability concerns. On the sustainability aspect, since FBS can only be sourced from accidental deaths suggesting that the supply of FBS is not always constant, causing fluctuations in the price and availability of FBS. Due to its high cost, FBS hinders researchers with lower budgets, limiting the locations where advancements in biology can take place. Secondly, it is highly unethical to kill pregnant cows as it is vital to consider the welfare of animals and treat them with respect and compassion.

Process of manufacturing FBS

There are six major steps in the production of FBS. When a pregnant cow is accidentally slaughtered, the fetus Is kept in the sterilized environment of the womb, and blood is extracted via a needle. This step can only occur in government approved slaughterhouses to prevent uncontrolled slaughter of the fetus. The collected blood is refrigerated to induce coagulation. When the blood is coagulated, the serum gets separated after centrifugation in order to removes clotting factors and cellular components from the blood. After this process, we are left with a highly pure serum with an orange hue. Then, the serum is filtered through 0.1 μm triple filtration, a filtration chain and is sometimes treated with irradiation (using gamma rays) (Lee et al., 2022). Finally, the serum is stored in sterile packaging and frozen until further use. This serum can be stored for an average of 5 years (Muniaraj et al., 2007).

Why is FBS Hard to Replace

Firstly, the chemical makeup of FBS is not fully discovered yet, meaning it is not possible to make an exact replica of FBS from synthetic components. Additionally, many of the enzyme, growth factors and hormones in FBS are very expensive to reproduce and cannot be mass produced. Even if some viable replacements are available, researchers are more likely to use FBS as it is widely accepted by the scientific community (Lee et al., 2022). Using FBS also results in the best cell proliferation, therefore giving more cells to work with to carry on the research.

Current Alternatives for FBS

Currently, there are many FBS alternatives such as FBM™, TesR™, Essential 8™, XerumFree™, Lipogro™, and okara extract. The problem with many of these alternatives is that it is hard to produce, therefore the cost is still an issue. On top of that, they are not as effective (Kolkmann et al., 2019). Although okara is cheap and easily obtainable, it still performs at a much lower level than FBS when measuring cell proliferation during a 14-day period (Teng et al., 2023). According to Figure 1, Primary myoblast growth in Alternative growth mediums peak in cellular proliferation at 30% of FBS's average performance. Since the chemical composition of FBS is not fully defined, any artificial serum trying to replicate FBS exactly will have a lower success rate.

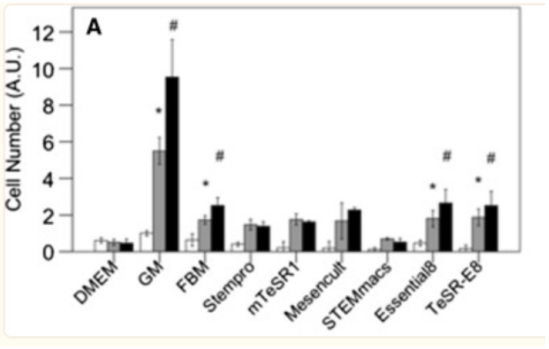

Figure 1. This is a bar graph comparing FBS (labeled GM) with artificially made growth media. The cells used to collect this data were primary bovine myoblasts. The y-axis contains arbitrary units used to relatively compares the success of the growth mediums (Kolkmann et al., 2019).

Why is milk a viable alternative?

Milk is an animal product just like FBS; therefore, it naturally has more protein content, which is shown to be an important factor in cell proliferation. Milk also has other important factors that contribute to cell growth, such as albumin, which are hard to find in synthetic alternatives. During the first few months in a mammal's life, milk is vital for growth (Foroutan et al., 2019).

In a recent study done by Muniaraj et al. (2006), the authors demonstrated that the use of goat milk with the addition of Media 199, a serum free supplement, is more effective than FBS in culturing *Leishmania donovani* Promastigotes by more than 40%. The high content of protein,

glucose, calcium, and triglycerides is hypothesized to be the cause of such growth (Muniaraj et al., 2006).

Section II: Specific Aims

FBS is very expensive and has an unstable production, which hinders underprivileged communities and public education systems from having wide research opportunities. Artificial synthetic alternatives, on the other hand, have a stable production, and stable pricing, but are still very expensive. The overall aim of this project is to engineer a milk-based mixture that is as versatile and effective for cell cultures. The final product will be a mixture of cow's milk, goat's milk, and other serum free additives, such as Media 199, which will have sufficient nutrients and macromolecules to enable cells to proliferate. The effectiveness of the growth media will be tested by growing a human immortal cell line, a primary chicken cell line, and a mammalian lactate cell line.

The primary goal of this project is to find an alternative to Fetal Bovine Serum that is at least 80% of the effectiveness of FBS and is budget friendly. To do this, 3 specific aims were set:

Specific Aim 1: Make 2 growth mediums, one with cow milk and one with goat milk, add serum-free additives and analyze the growth.

Specific Aim 2: Take data from the previous step and use it to create pairings of milk and FBS to minimize cost and maximize effiency.

Specific Aim 3: Refine the method and repeat the experiment with different concentrations of milk-based growth media.

Section III: Project Goals and Methodology

Relevance/Significance

Cell cultures are paramount in biological research. Some fields of study that rely on cell cultures are: drug development (drugs are tested on cells before humans), disease studies (understanding causes of diseases and potential biomarkers), vaccine production, toxicology studies, cancer research, tissue engineering/ regenerative medicine, and cellular agriculture (Segeritz & Vallier, 2017). For example, a research study will include studying cells will require a cell line to be subcultured for at least a month. For the study to be statically significant, there should be at least 5 plates of cells culturing simultaneously. If 3T3 cells, embryonic mouse fibroblast cells, are used in this research, the subculture process will have to be performed at least 50 times. Using a standard subculturing process for 3T3 cells, at least 135 ml of FBS is needed which will cost over $200. Instead, if FBS was substituted with bovine or goat milk, the cost will come to about $4. In this example, using milk instead of FBS will reduce the cost by 98%.

Innovation

Most growth media, including serum free media, have a complicated production process and are very expensive. With my project, growth media will be significantly cheaper than the current cost, and labs will be able to produce them instead of purchasing them from outside sources. The benefit of crafting a growth media in labs that is inexpensive, ethically sourced, and as effective as FBS can provide multiple research opportunities for low budget researchers, public schools, and even save money in regular research laboratories.

Methodology

1) Collection and treatment of milk
    a) Collect milk samples from a goat and a cow in strike containers
    b) Bring samples to lab and store them at a cold temperature
    c) Bring them back to room temp, boil it for 30 minutes, get it back to room temperature and freeze it again
    d) Continue the step above once a day for three days(tyndallization)
    e) Centrifuge the milk for 30 mins at 3000 rpm to remove the fat globules
    f) Collect the fat free milk and store it in a refrigerator
    g) Supplement the milk with a serum free additive
2) Methods to treat the cells
    a) Centrifuge the cells at 4000 rpm or 30 mins
    b) Wash the cells twice with sterile phosphate-buffered salt solution
    c) Adjust the cell count to 1 × 106 cells/ml using a Neubauer counting chamber.
    d) Filter sterile medium 199 with 25mg/liter gentamicin through a 0.22 micron filter membrane
    e) Inoculated 100 microliters of the cell solution in tubes with different culture media compositions
    f) Incubate tubes and monitor growth daily for 14 days
    g) subculture every other day
3) Methods to collect data:

- Take a small sample of the cells and put it under a hemocytometer to get the density of the cells in the GM

Specific Aim #1

The objective is to culture three types of cells with the growth medium derived from cow milk and goat milk.

Justification and Feasibility. The tyndallization and the centrifugation of the milk is necessary to eliminate unwanted components in the milk. Tyndallization is a heating process that will kill bacteria in the milk so the cells won't become infected. Although pasteurization is a more common practice of filtering milk, it denatures more proteins which is detrimental in the cell culturing process. The purpose of centrifugation is to remove the fat prevalent in milk. The processed milk will be stored in a refrigerator at -80 degrees to minimize any chemical reaction that might occur which will eventually spoil the milk. Unfortunately, milk doesn't have enough nutrients to support cell growth by itself, so several serum free additives will be used.

The cells will first be pre-cultured using FBS and then centrifuged to isolate from the FBS. The cells will be washed with sterile phosphate-buffered salt solution to remove any remaining FBS. The cell density will be adjusted to 10^6cells/ml which will ensure that there aren't too many cells when culturing, so the cells will grow properly and are not affected by contact inhibition. Cells will be inoculated with the milk-derived growth media and grown over a period of 14 days in an incubator (36 degrees, 5% CO2). The cells will be subcultured every other day to ensure enough space to grow.

Expected Outcomes. This knowledge will determine which growth medium (supplemented with FBS or milk) is the most efficient in cell proliferation. The expected outcome is that the goat milk-based growth medium will perform the best followed by FBS and

then the cow-based growth medium if milk has the same effect on mammalian cells as it has on *Leishmania donovani* Promastigotes.

Potential Pitfalls and Alternative Strategies. If neither of the milk-based mediums do not work, the cultures will be thoroughly examined to spot any contamination. If there is no contamination and the milk-based growth medium does not work, one of the following will be done: tyndallize the milk at a lower temperature to reduce protein denaturing, pair the milk-based growth media with FBS, or try the same process with a different cell line.

Specific Aim #2

The second aim is to collect and analyze the data from the previous and determine if milk needs to be supplemented by FBS and in what concentration. The data collection process will include two aspects: testing the cells quality (testing the function of the cell) and testing the quantity of the cells.

Justification and Feasibility. A cell count will be taken every other day to gather the data for cell quantity using a hemocytometer. Seven microliters of the cell culture will be injected into the hemocytometer and placed under a microscope. A hemocytometer has a specific volume and defined grid lines, which makes it easier to count for the researcher and gives fairly accurate results. To reduce human error in this process, there will be two people who count until both the people get the same number.

Expected Outcomes. The data from the hemocytometers will yield a mean of how many cells grew from each growth media. With this data, a 2 sample t tests will be performed to compare milk-based growth media to FBS. The first 2 sample t tests will compare the mean number of cells that grew with FBS to mean number of cells that grew in the goat milk-based

growth media. Another 2 sample tests will be performed to compare the mean number of cells that grew with FBS to mean number of cells that grew in the cow milk-based growth media.

The data from the assays will yield a percentage of the cells that are still functioning properly. With the percentages, two proportion z tests can be performed to compare the proportions of cells surviving from the milk-based growth media to the proportion of cells that survived in FBS. The first two proportion z tests will compare the proportion of cells that survived in the FBS growth media to the proportion of cells that survived in the goat milk-based growth media. The second two proportion z test will compare the proportion of cells that survived in the FBS growth media to the proportion of cells that survived in the cow milk-based growth media.

Potential Pitfalls and Alternative Strategies. If there are outliers in the data, the statistical test cannot be performed. An alternate strategy is to consider analyzing the growth trends over time as it can provide insights into the dynamics of cell proliferation and quality.

Specific Aim #3

The third aim is to refine the experiment and get a more optimal result by changing the concentration of the milk-based growth media.

Justification and Feasibility. There will be multiple variations that will be performed depending on the results. If the milk-based media show minimal growth, the second iteration will have double concentration of the milk-based media. If the milk-based media does not show any signs of growth, the milk will be supplemented with a small amount of FBS. This adaptive approach emphasizes continuous monitoring and iterative adjustments, ensuring a dynamic experimental process.

Expected Outcomes. Both-iterations should produce a higher number of cells, if not the same. Doubling the concentration of milk in the growth mediums might not have a change in cell proliferation if the cells reached their maximum potential. Adding milk to FBS might result in the same outcome. The benefit would be that if adding milk to FBS is just as effective as FBS itself, less FBS would need to be used in culturing cells, further reducing costs.

Potential Pitfalls and Alternative Strategies. Trying to refine the experiment for better results has some challenges and alternatives. Simply doubling the concentration of the milk-based growth media might not increase cell growth if they've reached their limit. Similarly, adding milk to FBS may not boost growth if the cells don't respond well.

Section IV: Resources/Equipment

Equipment

Equipment and resources that need to be purchased include 100ml of raw goat milk and raw goat colostrum, glutamax (to supplement the milk), Fetal Bovine Serum, sterile phosphate-buffered solution, and penstrep. Resources and equipment. used in a lab include a boiler, a -80C freezer, a centrifuge, hemocytometer, a 0.22 micron filter, 42 well microplates, a sterile environment and an incubator. Some other resources that will be used include distilled water, pipettes, and safety equipment such as gloves, masks, and lab coats.

Section V: Risks and Ethical Considerations

FBS contains proteins, and some individuals may be allergic to these proteins which can be a concern for researchers and laboratory personnel who come into contact with FBS. This risk can be combated by using personal protective gear.

Prolonged or improper use of gentamicin can contribute to the development of bacterial resistance, which is a concern in both clinical settings and research laboratories (Rouveix, 2003). Resistance can be prevented by following the minimum inhibitory concentration (MIC) of gentamicin. Avoiding using higher concentrations than necessary to minimize selective pressure for resistance (Rouveix, 2003).

In the process of tyndallization, the person performing the process will have to get close to and handle very hot and cold liquids. Individuals performing the procedure should be equipped with appropriate personal protective equipment, including heat-resistant gloves, safety goggles, and a lab coat, to safeguard against potential hazards.

High-speed rotation of the centrifuge can result in mechanical hazards. A malfunction, imbalance, or failure of the rotor can lead to equipment damage, and in extreme cases, it may cause the centrifuge to break apart. Improper loading or unloading of samples into the centrifuge can lead to physical injuries, such as finger injuries or hand entanglement with rotating parts (Grossel, 2005). This risk can be resolved by ensuring the centrifuge is properly locked and it is properly plugged. An additional safety measure can be taken by maintaining a proper distance from the centrifuge so even in the case of a malfunction, no harm is done.

Raw milk can harbor harmful bacteria, including Escherichia coli (E. coli), Salmonella, Listeria, and Campylobacter. These bacteria can cause serious illnesses, leading to symptoms such as diarrhea, abdominal cramps, and, in severe cases, kidney failure or death (Berge &

Baars, 2020). To resolve this danger, the milk will be stored in a sealed container until the tyndallization process is over. Every piece of equipment that makes contact before the tyndallization process is over will be thoroughly washed. Gloves, goggles, and masks will be used in every step of this process.

Section VI: Timeline

Aug.		Sept.				Oct.				Nov.				Dec.				Jan.				Feb.	
3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2
Phase 1: Background research																							
						Phase 2: Proposal/ finding lab																	
												Phase 3: Testing and refining											
															Phase 4: Analysis									
																		Phase 5: Presentation					

Phase1:

- Read 20 research articles (at least)
- Find 3 patents/ competitors
- Understand how to execute my experiment
- Find a mentor

Phase 2:

- Revise project with mentor
- Join a lab
- Collect preliminary data
- Analyze preliminary data
- Refine details of my experiment

Phase 3:

- Pre-culture all the cell lines needed with FBS
- Use different GM and observe which is doing the best
- Refine and make a better replacement after a week
- Repeat this again

Phase 4:

- Analyze the data to refine different Growth Media

Phrase 5:

- Make the poster

Section VII: Appendix

Section VIII: References

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