

Full Length Research Paper

Development of a gas exclusion technique to sterile procedures in non-sterile environment with application to the biopsy of cartilage

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Abstract

Nowadays, the most promising form of treatment for patients with advanced forms of articular cartilage degeneration, under active development is the Tissue Engineering solution. The extraction of the required samples for effective TE research is complicated by the absolute need for sterility during the biopsy procedure. But there are situations in which such a biosafety hood is not able to accommodate the required operation, particularly in the case of large subject matter which will not fit within the hood without disturbing laminar hood flow, also in the case where large pieces of equipment such as powered tools are required. So the necessity of a simple and easily accessible method has been felt. This project was based upon the development and validation of a technique. The basic concept to be developed is to use a pre-existing source of sterile nitrogen gas to produce an enclosed zone of positive pressure at the region of dissection, in a way that any pathogens from the region are carried away from the incision and any pathogens in the surrounding area are prevented from entering via diffusion by means of a dominant counter-active convection. After the explants were extracted by this method, in order to confirm that the proposed nitrogen gas delivery system is bacteria free, sterility test were performed. These experiments were repeated two times per week during three weeks. In total for 48 samples which were extracted from 12 synovial bovine joint (each joint four samples). The results of sterility test show no colony formation which means there is no infection in this technique at all. Also this method was very safe and does not have any biohazard, respiratory hazard and it complies with material safety requirements. Successful validation of this technique may also allow application within emergency medical treatment outdoor surgery and agricultural veterinary care.

Keywords: Synovial joint, sterile non- sterile, tissue.

INTRODUCTION

Articular cartilage

The movements necessary for essential activities of everyday life, work, and recreation are made possible by the action of synovial joints. These units provide painless stable movement for living things, with a low level of friction, and are usually able to function for a lifetime. The extraordinary functional capacities in these units particularly derive from articular cartilage, the tissue that

forms the bearing surface of every synovial joint (Buckwalter et al., 1988; Buckwalter and Mankin, 1997; Buckwalter, Rosenberg, and Hunziker, 1990). A specialized connective tissue, it both bears the load through the joint and reduces friction at the point of articulation. Although it is only a few millimeters thick, it has wonderful resistance to compression, considerable elasticity, high durability, and a surprising ability to minimize the stress levels transmitted to the subchondral bone (Buckwalter and Mankin, 1997). Unlike other kinds of tissue, cartilage tissue has a low level of metabolic activity; therefore, it has very little response to any

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change caused during loading or injury. Although it has a low level of metabolic activity, a deeper study of the morphology and biology of this tissue reveals a wonderfully complex and ordered structure, with various multiple interactions between the chondrocytes and the maintenance matrix of the tissue (Buckwalter and Mankin, 1997; Buckwalter, Mow, and Hunziker, 2001).

Articular cartilage disease

The specific structural organization and molecular composition of this tissue gives it the particular biomechanical properties that make it possible to act under the difficult conditions associated with demanding physical activities (Buckwalter, et al., 2001). However, when articular cartilage is damaged, not only does it not heal, but it also often degenerates further; a degeneration that will lead to pain and loss of function of the joint (Gevork, 2008). Nowadays, despite many advances in treatment for patients with advanced forms of articular cartilage degeneration, the reconstruction of a state equivalent to that of healthy undamaged cartilage remains to be achieved. The most promising form of treatment under active development today is the Tissue Engineering solution, essentially comprising the biopsy of cartilage, the isolation and expansion of the cells therein, the placing of those cells within a scaffold material, and the re-implantation of the scaffold to affect a fully biological repair.

Tissue engineering for articular cartilage repair

Tissue engineering has been employed as an alternative method for the repair of defective articular cartilage (Lohmander, 2003). Several surgical procedures for cartilage treatment, some of which involve tissue transplantation, have been performed by clinics in recent years. One of these procedures is termed autologous chondrocyte implantation (ACI), a process made possible by the isolation of autologous articular chondrocytes by biopsy from excised non loading-bearing tissue and subsequent in vitro expansion in an appropriate culture medium (Brittberg, 2008).

There are three main constituents which generally form the basis of tissue engineering for cartilage: A cell source; A signaling strategy (both biochemical and/or mechanical and appropriate scaffold material (Dario and Fauza, 2003).

Challenges in getting sources for articular cartilage tissue engineering

Unfortunately, the extraction of the required samples for effective research is complicated by the absolute need

for sterility during the biopsy procedure. Explants should be dissected using an appropriate protocol to develop and avoid contamination and retain cell survival. The level of sterility needed is somewhat in excess of that required for routine surgical operations, because in this case biopsy must survive incubation in a high growth medium for some time, in the absence of the immune system. Different types of culture contamination exist, such as biological or chemical, seen or unseen, destructive or seemingly benign. The quality and function of cell cultures can be affected unfavorably by any kind of contamination, which in general eventually results in cell death (Ryan, 2008). Culture loss due to contamination is a universal problem which has been experienced in culture laboratories and by cell culture workers.

Even a trace of bacterial, viral or fungal pathogen is likely to grow unabated until the biopsy must be disposed of. For this reason it is usual that, in the research context, the biopsy is undertaken within a sterile laboratory environment, usually by means of a class II biosafety hood equipped with microbiological filtering and laminar flow system. Regrettably there are situations in which such a biosafety hood is not able to accommodate the required operation, particularly in the case of large subject matter which will not fit within the hood without disturbing laminar hood flow, but also in the case where large pieces of equipment such as powered tools are required for specific forms of biopsy procedure.

Historical setting

Over a hundred years ago Pasteur, Koch, Lister and other pioneer microbiologists and surgeons founded that the main reason of infection in wounds is bacteria. So, it is clear that for preventing infection in hospitals and operating rooms the elimination of bacteria is necessary, this became the scientific basis for the first clean rooms.

In 1864, Sir John Simon proposed that the ventilation must „flow from inlet to outlet“ because it is more effective for removing the airborne contamination from hospital rooms, leading to airflow that will be applied by an artificial system.

In 1946, Bourdillon and Colebrook investigated the idea of using filtered air for a dressing station. They discussed the possibility of using 60 great air changes per hour. They considered the „piston effect“ for making a draught and pushing the dirty air to the outside. The result, according laboratory tests, was a „sudden disappearance of a bacterial cloud“.

In 1960, Blowers and Crew described downward displacement of air with a minimum of turbulence. The pattern of artificial ventilation became widespread in hospitals in the 1960s.

Conventional turbulently ventilated rooms were addressed by consideration of the type and placement of

air diffusers and exhausts, the influence of a temperature difference between incoming and ambient air, the effect of air supply volume on the dilution of airborne contamination, air filter efficiency and air movement control between areas.

The main impetus for improving the air conditions in the operating room came from Professor Sir John Charnley's idea in the early 1960s: He fundamentally improved the design and technique of setting of an artificial hip joint. It was efficient operation but the joint sepsis rate at the beginning of his studies was almost 9%. At that time methods of treatment the infection were not effective and it resulted in having to remove the artificial joint. According his theory, it was because of airborne bacteria.

In the 1980s the development of ultra-clean operating room systems was mounted by the medical research of the United Kingdom Council and their results were a vindication of Charnley's work. The pattern of cleanrooms was used in operating rooms.

However it was soon appreciated that „bacteria-free“ was not the same as „particle-free“. A great deal of effort was therefore put into ensuring that materials and surfaces did not generate particles, but it was not fully appreciated that airborne dispersion of large quantities of particles by machines and people had to be removed by large quantities of pure air so the application of cleanroom industry was expanded and diversified (Whyte, 1991).

The sterile field and laminar flow hood; microbial air filtering

The growth rate of bacterial or fungal cells is usually so much greater than that of mammalian cells that no level of contamination can be tolerated in an animal cell culture. It is impractical to design a laboratory that is totally free of potentially contaminating microorganisms so it is generally attempted to decrease the contamination rate.

The airborne contaminations rate will be reduced eventually by using the laminar flow cabinet in the laboratory (Butler, 2004).

Sterile operations in a cell culture laboratory are normally undertaken in cabinets (commonly called „hoods“), which serve the purpose of minimizing the chance of culture contamination and ensure the safety of the operator.

The use of human or other primate cells provide the transmission of infectious agents so some protections are required. Open-fronted laminar flow cabinet supplies spaces which only operator's arms enter the sterile environment and vertical flow of air filtration and horizontal working surface maintain security during biological research (Doyle and Allner, 1990).

For working with low-to-moderate toxic or infectious

agents, a class II cabinet is necessary. In this type of cabinet the operator can enter his hands but his breathing is hindered by a Perspex cover over the working area.

A high-efficiency particulate air (HEPA) filter provides airflow into the cabin. The design of HEPA filters serves the purpose to capture inessential airborne particles or aerosols. They are assembled from an uninterrupted sheet of submicron glass fiber folded back and forth over a corrugated spacer as a support. The common HEPA filter in a class II cabinet ensures a 99.99% efficiency of entrapment of 0.3 μ m particles.

Most of the air (70-80%) is recirculated to form an air curtain which serves to maintain sterile space for culture manipulation. The air curtain formed in the internal front face of the cabinet has a typical flow rate of 0.4 m/s. The use of a HEPA filter makes for a safe laboratory environment free from any possible pathogens or toxic elements (Butler, 2004).

Summary

Contamination control is the most important target in tissue or cell culture laboratory. One the universal problem which has felt by tissue culture laboratories and cell culture workers is culture loss due to contaminations. Between the different types of culture contaminations biological contaminations such as bacteria, fungi and yeast are the most common contaminations on cell culture. These kind of contaminations can be grow very fast over the medium and shift in pH of the culture which will result in media color change and can be detect visually by regularly control. The result of appearance of non-cellular material will be dissolution of cells by lysins and finally cells death. Some consequences of contaminations are loss of time, money and effect incorrect experimental results and adverse effects on the culture, losing the valuable products and finally personal embarrassment.

Although the preventing of contamination has long been dreamed of by researchers, completely reduction of contamination is not possible. The practical solution of this important problem can be achieved by some strategies such as use good aseptic techniques and maintaining sterility, keep the laboratory clean and free of dust, monitoring for contamination routinely and using of antibiotics if it be necessary.

Among of different strategies in order to decreasing contaminations using the laminar flow hood cabinet have been had ideal results. It can be reduced eventually the airborne contaminations rate (Butler, 2004). Sterile operations in a cell culture laboratory are normally done in laminar flow cabinets because the air flow through the sterile exhaust of the cabinet can minimized amount of particles in the environment. Unfortunately this solution cannot be useful and practical when large pieces of

tissue or explants are needed for tissue engineering experiments or treatments. In order to solve this problem the main aim of this project was to use a pre-existing source of sterile nitrogen gas to produce an enclosed zone of positive at the region of dissection, in a way that any pathogens from the region are carried away from the incision and any pathogens in the surrounding area are prevented from entering via diffusion by means of a dominant counter-active convection. We have used nitrogen because Nitrogen is generally uncreative at standard temperature and pressure. According its properties, especially the inertness property, Nitrogen has various applications in different fields and industries. It can be used as an inert replacement of air specially where there are unwanted oxidation conditions (Kerbs, 1998).

In the group 15 of the periodic table nitrogen is the lightest element. Nitrogen as an element, forms many compounds but as a diatomic molecular gas (N_2) is not very reactive (Kerbs, 1998).

RESEARCH METHODS

Articular cartilage explants harvesting

Fresh articular cartilage should be used for this project in order to obtain more viable cells. Therefore, bovine feet are obtained on day of slaughter from a local abattoir. Open arrival to the laboratory, feet are washed, and immediately afterward the feet were put into alcohol (Ethanol C_2H_5OH) 70 % (v/v), for at least 15 minutes.

Each foot was put in a stainless steel tray and sprayed with alcohol, then transferred to a sterile laminar hood (class II) in order to remove the skin of the foot, and opening the synovial capsule and exposing the articular surface. At all times, touching of the articular surfaces should be avoided. The exposed metatarsus was wrapped with aluminum foil which was previously sprayed with alcohol before being brought into the laminar hood. This was carried out to ease handling of the joint as well as to keep it clean (Figures 3.1 -3.3).

In order to transport the metacarpal-phalangeal joint out from the class II safety cabinet, the joint was placed in an autoclaved plastic bag (Figure 3.4) together with a sterile universal container. Before tying the plastic bag, one end of a sterile plastic pipe (approximately 90-100 cm length and 7-7.50 mm diameter) was placed inside the plastic bag while another end was left free (Figure 3.4). The open end of the pipe will be used to allow the transfer of nitrogen gas to the joint. The assembly was then transferred from the clean laminar hood to the open air.

The design and set-up of gas exclusion system

The main purpose of the whole study was to develop a

system that allows extraction of articular cartilage explants outside the Class II laminar hood. Ideally, this extraction should be carried out in the laminar hood. However, because the extraction involves a great deal of forceful of hammering into the bone which will damage the base of the laminar hood, a surrogate system was to be built outside the laminar hood. Nitrogen gas was chosen as a part of the design because this gas is known to be an inert and non-toxic gas (other than by oxygen exclusion). The details of the setting up of the system are presented below.

Nitrogen gas delivering system

A glass bubble column including two coaxial glass columns was constructed; the outer glass column has a diameter of 4.5 cm and is 45 cm in length while the inner glass column has a diameter of ~1 cm and is 40 cm in length, as shown in Figure 3.5. The inner column was placed in the center of the outer column. The inner column was open on both top and bottom. The lower head of that was designed in shape of a net in order to increase contact surface. The inner column has two openings; one end is known as the input terminal where nitrogen gas will enter while the other is attached to a specially made end which was placed in the center of the outer column. Commercially obtained compressed nitrogen gas is a very dry gas, and if directly exposed tissue causes rapid dehydration. To avoid this taking place, the bottom end of the inner column bottom was attached to this coaxial column. The outer glass bubble column has only one opening which is known as the output. The rehydrated nitrogen gas will exit through this opening. Both terminals were designed to fit the autoclavable plastic pipes.

Nitrogen gas enters through the input terminal, and then passes into the inner column. It then bubbles upwards through the water, accumulating moisture in external column as it moves toward the top and goes out from the external terminal (Figure 3.5).

The role of glass bubble column is to prepare the circulation of nitrogen gas, in order to wet the gas.

Experimental setting up

A retort clamp stand was used to hold the nitrogen gas delivery system. The glass bubble columns were autoclaved before use. The outer glass column was filled up with autoclaved distilled water (approximately 10 cm elevation from the bottom of the outer column) in this state the lower head of the inner bubble column was immersed in water this is to make sure the specially made filter is totally immersed in the water. The outer glass column was placed in the bathwater (with an approximate temperature of 37°C) to warm up the nitrogen gas. The purified nitrogen gas is a very cool and



Figure 3.1 Blades were used to exposed the synovial capsule of the joint



Figure 3.2: Exposure of the joint surface from a metacarpal-phalangeal joint



Figure 3.3: Covering the long cannon bone with aluminum foil

dry. Although is not shown in this study, previously by direct exposure of the joint to nitrogen gas caused serious dehydration of tissue, as well as rapid cooling. Allowing the nitrogen gas to mix with water can help to rehydrate the nitrogen gas and aid thermal equilibrium with the surrounding environment. The rehydration and warming of the nitrogen gas were the main reasons why the nitrogen gas delivering system was built.

The metacarpal-phalangeal joining, which was wrapped in an autoclaved plastic sheet (Figure 3.4), was placed in a clamp (size 6) and tightly secured. The loose end of the pipe from the bag was connected to the output terminal of the outer glass column (Figure 3.6). All necessary equipment, such as a hammer, punch, scissors and forceps were autoclaved prior to use.

The purified nitrogen was allowed to flow into the



Figure 3.4 Metacarpal-phalangeal joint, sterile universal container and sterile plastic pipe was placed inside the autoclaveable plastic. A cable tie was used to tighten the bag. One end of the pipe was left free.

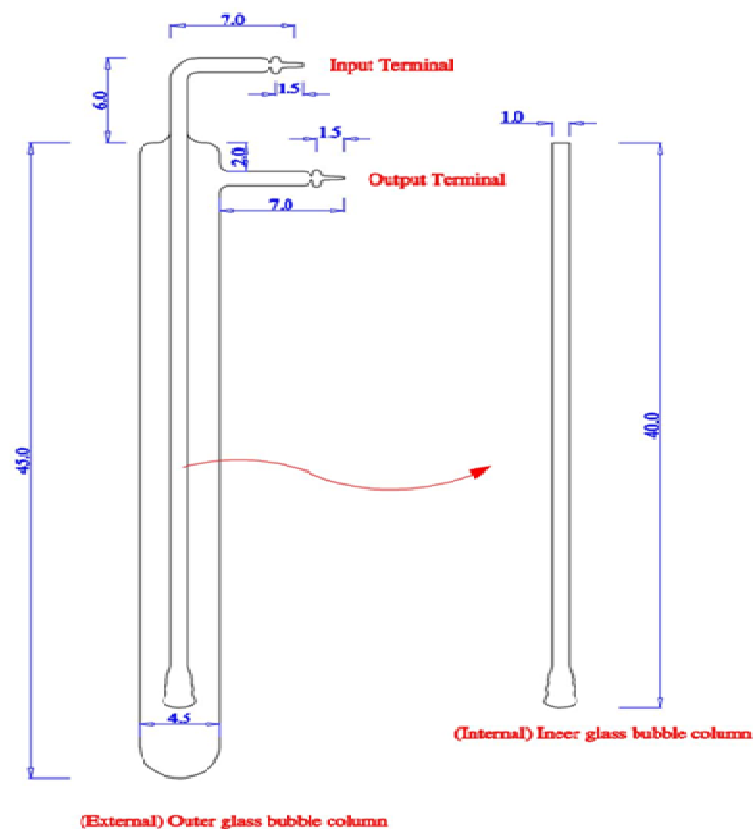


Figure 3.5: A schematic design of a nitrogen gas delivering system

glass bubble column once the setting up was completed. The autoclaved bag was inflated with humidified nitrogen gas which caused the expansion of the bag. A small hole was created on the plastic bag in order to place the punch and forcep into the plastic bag (Figure 3.8). The

punch used was 1.75cm in diameter and 10 cm in length (Figure 3.7) and this punch was specially designed for the experiment. The plastic bag stayed inflated because there was a constant supply of nitrogen gas, and this pressure can be felt in the resistance of the bag to



Figure 3.6. The setup of the system. The metatarsus was tightly clamp while the loose end of the pipe from the autoclaved bag was connected to the output terminal of the outer glass column



Figure 3.7. A specially designed punch was made for this experiment in order to extract explant

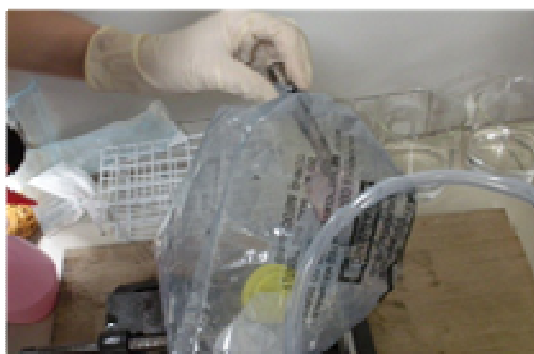


Figure 3.8. During the filtration of nitrogen gas into the autoclaved bag, a small hole was created to fit in the punch.

crumpling. The hammer was placed on top of the punch and a great force was exerted on the metacarpal-phalangeal joint surface (Figure 3.9).

The articular cartilage explant was retrieved by using

a sterile forceps which was previously put into the bag and the explant was put into the sterile container which was already inside the plastic bag (Figure 3.10). About 4 explants were extracted from a joint. At the end of the



Figure 3.9 The hammer was placed on top the punch in order to create an articular cartilage explant.



Figure 3.10 Using forceps, an explants was put into the sterile container previously placed within the bag

experiment, the container cover is tightened and transferred to the laminar hood. This is followed by a series of sterility tests.

RESULTS

In order to confirm that the proposed nitrogen gas delivering system is bacteria free, sterility test were performed. Sterility tests are one of the main diagnostic methods used in microbiology and bacteriology and involve attempting to grow and culture microorganisms in an artificial environment.

After the explants were extracted from the joint, the explants were placed in a sterile container and brought to the class II laminar hood. Explants were washed with distilled water. After washing the explants, each explant was placed in an individual container containing PBS. Four explants were retrieved from every joint. In total 48 explants were used in these experiments, each experiment was repeated twice.

In order to allow the microorganisms to grow, the

growth media or agar was inoculated with a sample and then put in the incubator. The plates were checked visually for colony formation. Four different kinds of agar were used as culture media in this study. These agars are Chocolate Agar+ Horse Blood which is suitable for the isolation and cultivation of a variety of fastidious microorganisms, MacConkey Agar that is used for the selective isolation, cultivation and differentiation of coliforms and enteric pathogens based on the ability to ferment lactose. Sabouraud Dextrose Agar suitable for the cultivation of pathogenic and nonpathogenic fungi, especially dermatophytes. Columbia Agar With 5% Horse Blood that is suitable for isolation, cultivation and detection of hemolytic activity of streptococci, pneumococci and other particular fastidious microorganisms (Figure 4.1).

By using a sterile plastic loop the medium containing cartilage explant was correctly spread over the surface of the agar (Figures 4.2, 4.4, 4.6, 4.8).

After inoculation, the agar plates were left inside the incubator (37°C and CO₂) for at least 48 hours in order



Figure 4.1. Different types of Agar plates used for sterility test



Figure 4.2. Chocolate Agar + horse Blood plates after inoculation by sample

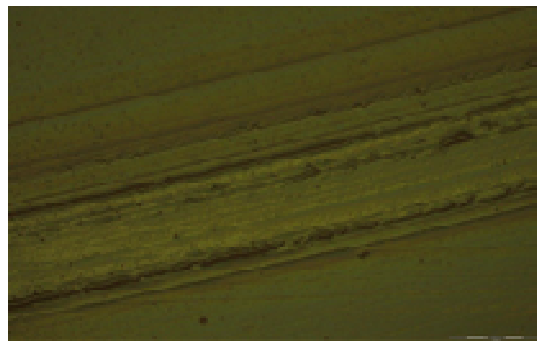


Figure 4.3. Microscopic photographs Chocolate Agar + Horse Blood plates after incubation, photograph are showing there is no colony formation at all

to allow the microorganisms to growth. The agar plates were removed from the incubator after 48 hours to check for colony formation. The results of observations have demonstrated in tables (4.1-4.4)

The sterility test was performed for each of eight samples extracted from two different bovine synovial joints at different times in one day. These experiment were repeated 12 times and all the results shown no colony formation (Figures 4.3,4.5,4.7,4.9).

Samples in open environment imcontrol study

For the control studies, the articular cartilage explants were extracted in the open air. Explants were placed in a sterile container and washed in PBS. By using a sterile plastic loop the medium containing cartilage explant was correctly spread over the surface of the agars. After inoculation, Chocolate Agar + horse Blood, MacConkey, Sabouraud Dextrose, and Columbia Blood Agar with 5%

Table 4.1. Chocolate Agar + Horse Blood Plates after Inoculation by Sample

| Number of Sample | Colony Formation | Observation Duration |
|------------------|-------------------------|----------------------|
| One | Negative (no infection) | 48 hours |
| Two | Negative(no infection) | 48 hours |
| Three | Negative (no infection) | 48 hours |
| Four | Negative (no infection) | 48 hours |

Table 4.1. Observations Results of Mac Conkey Agar Plates for Colony Formation

| Number of Sample | Colony Formation | Observation Duration |
|------------------|-------------------------|----------------------|
| One | Negative (no infection) | 48 hours |
| Two | Negative(no infection) | 48 hours |
| Three | Negative (no infection) | 48 hours |
| Four | Negative (no infection) | 48 hours |

Table 4.3. Observation Results of Sabouraud Dextrose Agar Plate for Colony Formation

| Number of Sample | Colony Formation | Observation Duration |
|------------------|-------------------------|----------------------|
| One | Negative (no infection) | 48 hours |
| Two | Negative(no infection) | 48 hours |
| Three | Negative (no infection) | 48 hours |
| Four | Negative (no infection) | 48 hours |

Table 4.2. Observations Results of Columbia Blood Agar With 5% Horse Blood for Colony Formation

| Number of Sample | Colony Formation | Observation Duration |
|------------------|-------------------------|----------------------|
| One | Negative (no infection) | 48 hours |
| Two | Negative(no infection) | 48 hours |
| Three | Negative (no infection) | 48 hours |
| Four | Negative (no infection) | 48 hours |

**Figure 4.4.** MacConkey Agar plates after inoculation by sample

Horse Blood agars plates were left inside the incubator (37°C and CO₂) for at least 48 hours in order to allow the microorganisms to grow. The Chocolate Agar +

horse Blood, MacConkey, Sabouraud Dextrose, and Columbia Blood Agar with 5% Horse Blood agar plates were removed from the incubator after 48 hours to check

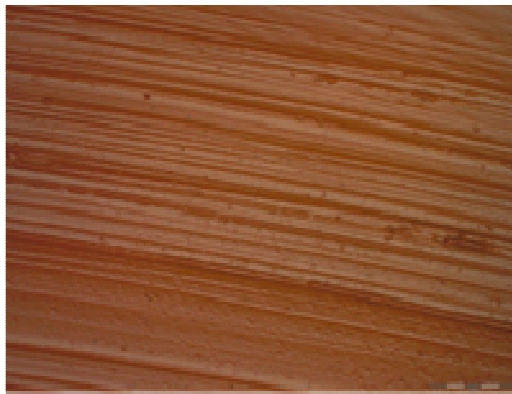


Figure 4.5. Microscopics photographs of MacConkey Agar plates after incubation, photographs are showing there is no colony formation at all



Figure 4.6. Sabouraud Dextrose Agar plates after inoculation by sample

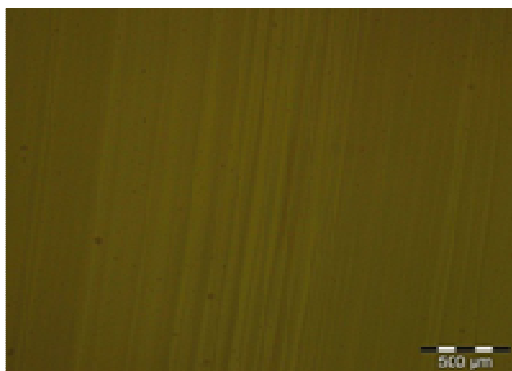


Figure 4.7 Microscopic photographs of Sabouraud Dextrose Agar plate after incubation

for colony formation. The results of observations have demonstrated in Figure 4.10a-d. Clearly, all the agars

have shown colony formation and this indicated the growth of microorganisms of explants extracted in an



Figure 4.8. Columbia Blood Agar with 5% Horse Blood

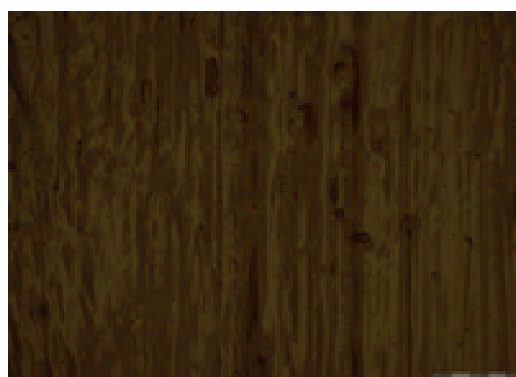


Figure 4.9. Microscopic photographs of Columbia Blood Agar with 5% Horse Blood plate after incubation. Photographs are showing there is no colony formation at all.

open air.

DISCUSSION

Medical technology has been progressed very fast in last few years. The treatment of patients with advanced forms of articular cartilage degenerations also has been included of these advances but the process of rebuilding to a state equivalent of a healthy undamaged cartilage remains to be achieved yet.

The significant advances in this subject has been achievable by using Tissue Engineering which has included of four main steps first the biopsy of cartilage, second the isolation and expansion of the cells therein, third the placing of those cells within a scaffold material, and finally the re-implantation of the scaffold to affect a fully biological repair.

The sterility is a major issue for successful research and treatment in this field. Because the absence of the immune system prepares suitable culture for

microorganism's growth so high level of sterility is required during the biopsy procedure.

Therefore the sterile laboratory environment is required. For this purpose usually a class II biopsy hood equipped with microbiological filtering and laminar flow systems have been used. But there are some problems in case of huge subject matter which needs large pieces of tools and equipments. The biopsy hood is unable to provide the required space for such operation.

Because of the importance of explants of articular cartilage for tissue engineering research and articular cartilage treatment the necessity of a simple and easily accessible method has been felt.

The purpose of this study is to develop a gas exclusion technique to allow sterile procedures to be conduct in a non-sterile environment.

Nitrogen gas was selected for this study because it is a diatomic molecular gas (N_2) which is not very reactive.

Since this project does not have any similar parable, we cannot compare it with other viewpoints. In this chapter therefore we have decided to note the important



Figure 4.10 a



Figure 4.10 b

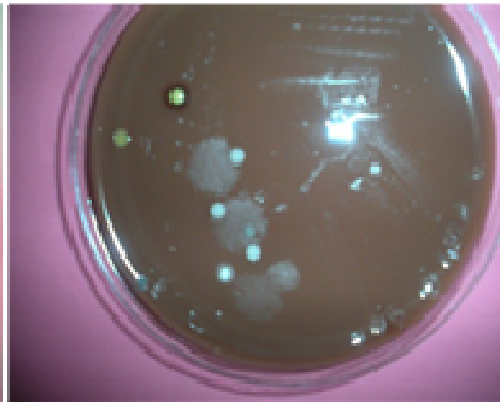


Figure 4.10. agar plates in open environment, a- Columbia blood agar + 5% horse blood, b- Sabouraud Dextrose Agar, c- MacConkey agar, d- Chocolate Agar + horse Blood

needs of developing a locally sterile environment setting technique. The results of this project are useful for routine surgical operation and biopsy when large pieces of tissue are used, for example, when samples for articular cartilage treatment or tissue engineering research are required.

The main purpose of this research was validation of a gas exclusion technique to permit makeshift sterile procedures in a non-sterile environment with specific application to the sterile biopsy of cartilage from articulating joint. We were hopeful by using nitrogen gas, which is an inert gas, in a closed area we can get large pieces of articular cartilage that is not possible to get in sterile environment of laminar hood. Luckily our technique was successful. The results of our experiment of 48 samples which were collected by this method showed no infection and contamination at all.

These samples after collection by this method were assayed by sterility test. For this purpose we used four different types of agar include the results of sterility test with these kinds of agars showed any infection which means absence of microorganisms in our pet

zone of experiment. Whereas when explants were collected in open environment and then the similar processes did for them the results of sterility test were exactly different and all the agars have shown colony formation and this indicated the growth of microorganisms of explants extracted in an open air.

This method has shown good results which means this method can be used not only in tissue engineering research and articular cartilage treatment but also in emergency medical cases such as military, emergency medical treatment and agriculture veterinary care.

Also this method is very safe and does not have any biohazards, respiratory hazards and cry hazards.

CONCLUSION

Around the joint surface can found particularly Articular cartilage, which is specialized as connective tissue. Bearing the loads and reducing friction across moving joints are two main duties of these joints. While it has only almost a few millimeters thickness, it has perfect

strength to compression, elasticity, high durability, and surprising ability to load with minimum level of stresses on subchondral bone (Buckwalter and Mankin, 1997). But when the articular cartilage damage, not only does not treat it, but more often degenerates; the pain and loss of joint function lead (Gevork, 2008). Currently even though many advances in treatment for patients with advanced forms of articular cartilage degeneration, the reconstruction of a state equivalent to that of healthy undamaged cartilage remains to be achieved. Developments and new approach to treat this kind of problem lead us to Tissue Engineering solution, essentially comprising the biopsy of cartilage, the isolation and expansion of the cells therein, the placing of those cells within a scaffold material, and the re-implantation of the scaffold to affect a fully biological repair. In the early of twentieth Tissue culturing was formed for the first time by Harrison in 1907 and Carrel in 1912. The original methods developed for tissue culture by Harrison and Carrel involved the maintenance of tissue fragments (or explant) on a solid surface and supplied with suitable nutrients. One of the major difficulties and reasons for failure at this stage is that the cell population becomes contaminated with bacteria or fungi. To avoid this problem it is important to maintain aseptic techniques throughout the process (Butler, 2004).

The aseptic techniques necessary for cell culture can now be performed easily in a well-designed laboratory and are far less extravagant than methods suggested by Carrel. The major source of pollution is airborne and arises from inadequate aseptic techniques and most often instigates from human contact such as from hands, breath or hair. The risk of contamination can minimize by the available equipment and cultural supplies. The risks can also be reduced further by careful attention to detail when handling cultures (Butler, 2004).

Growth of cells in a controlled environment such as a laboratory separately from its natural and original source called Cell culture or Tissue culture (the terms are used interchangeably). These cells can be cultured in vitro. Vitro has an entirely diverse environment as a result of the absence of histological organization. So the elements which were before active in natural environment no longer activates which will results by loss of the specific structural and functional attributes and ability such as immunity response to infections. Therefore the absence of contamination by the different micro-organisms is a crucial segment of all animal cell culture because the growth of cells in vitro is dependent on the complete aseptic environment (Sinha and Kumar, 2008).

In addition the extraction of the required samples for effective research is complicated by the absolute need for sterility during the biopsy procedure. Explants should be dissected out following an appropriate protocol to develop and avoid contamination and retain cell survival.

The level of the sterility needed is somewhat in

excess of that required for routine surgical operations, because in this case biopsy must survive incubation in a high growth medium for some time, in the absence of the immune system. Even a trace of bacterial, viral or fungal pathogen is likely unabated until the biopsy must be disposed of. Scientists are willing to remove all contaminations of cell culture but it cannot be totally eliminated, just it should be directed to reduce its rate and incident.

For this reason it is typical like in the research context, the biopsy is undertaken within a sterile laboratory environment, usually a class II biosafety hood equipped with microbiological filtering and laminar flow system.

Unfortunately there are situation in which such a biosafety hood is not able to accommodate the required operation, particularly in the case of large subject matter such as bone or articular cartilage which will not fit within the a scaffold material, and the re-implantation of the scaffold to affect a fully biological repair.

According to importance of this subject for tissue engineering research and particular treatment the necessity of a simple and easily accessible method have been felt.

Therefore, the main subject of our study was development and evaluation of a technique based on using positive pressure nitrogen gas in order to provide a locally and temporary sterile environment, which can be suitable in a clinical setting, general laboratory and also can be applicable in outdoor surgery, emergency application, agricultural veterinary care and so on.

This project was based upon the development and validation of a technique to be based upon atmospheric exclusion using positive pressure nitrogen gas in order that locally sterile environment might be produced even in general laboratory or clinical setting. we have choice nitrogen because Nitrogen as an element, forms many compounds but as a diatomic molecular gas (N_2) is not very reactive (Kerbs, 1998) it is a nontoxic gas and because of its inertness properties and its ability to be liquid in very low temperature has many uses. Successful validation of this technique may also be of applicability within emergency medical treatment outdoor surgery and agriculture veterinary care.

The basic concept to be developed was to use a pre-existing source of sterile nitrogen gas to produce an enclosed zone of positive pressure at the region of dissection, in a way that any pathogens from the region are carried away from the incision and any pathogens in the surrounding area are prevented from entering via diffusion by means of a dominant counter-active convection.

Core outputs of the project were:

Development and codification of a procedure for sterile

biopsy outside of a bio safety hood or other sterile environment. In order to test validation of sterility we have used the standard and internationally recognized form of test protocol. In this research we have employed incubation method for sterility test. In incubation method, the grow media or agar inoculates with a sample and then put them in the incubator in order to allow the microorganism to grow. Four different kinds of agar were used as culture media in our study. These agars were:

Chocolate Agar + Horse Blood, Mac Conkey agar, Columbia blood agar with 5% horse blood, saboraoud dexteros agar. The agar plates then will be checked visually for colony formation. The process have been done and repeated for more than 48 samples. These experiments were repeated two times per week during three weeks. In total for 48 samples which were extracted from 12 synovial bovine joint (each joint four samples). The results of observation for every 48 samples were the same. It is cleared there is no infection at all.

This method has shown good results which means this method can be used in tissue engineering research and articular cartilage treatment also because of the temporary and easy application of this technique it can be used in emergency medical cases such as military, emergency medical treatment and agriculture veterinary care in future.

Only Limitation of this technique is safety observing in using nitrogen gas. Although, nitrogen is a nontoxic gas but it can be an asphyxiant gas. Breathing the compress air including nitrogen and oxygen can cause very serious physical problem. If someone breathes pure nitrogen for a period of time will die; the reason of death is not because of the nitrogen gas but it is because of the depriving of the oxygen in body. Nitrogen is only slightly soluble in alcohol and water. It is an asphyxiant gas so it is named silent killer. If someone breathes pure nitrogen it will strip the body of oxygen, so observing the safety rules in usages of nitrogen gas is the most important subject for who are working by this gas. They can

decrease these hazards by wearing the mask and avoid of breathing the pure gas.

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