ASSESSING SOFT-EMBALMED CADAVERS AS A BIOLOGICAL HAZARD

by

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Abstract

At Queen's University Department of Biomedical and Molecular Sciences, as is the case at many Canadian institutions, three primary methods are used to generate three distinct cadaver types for human anatomical education: fresh/frozen, traditional formalin hard-embalmed, and phenol-based soft-embalmed. Each of these techniques has its own merits and restrictions, with phenol-based soft-embalming combining the longevity seen in traditional embalming with the life-like quality characteristic of fresh/frozen tissue. Current environmental health and safety (EHS) restrictions place traditional formalin embalmed cadavers in Containment Level 1 (CL1) laboratories, with both fresh/frozen and phenol-based soft-embalmed cadavers in Containment Level 2 (CL2) laboratories. However, there is a gap in the current literature dictating whether or not phenol-based soft-embalmed cadavers do in fact belong in CL2 laboratories. In this hypothesis-driven report, it was hypothesised that soft-embalmed cadavers are a higher risk biosafety hazard than formalin-embalmed cadavers, thus warranted for designation to CL2 laboratories. After an in-depth analysis of the present literature surrounding the use and properties of phenol-based soft-embalmed cadavers in comparison to traditional formalin hard-embalmed cadavers, it was found that soft-embalmed cadavers do become colonized more readily by micro-organisms, aligning with our hypothesis. To definitively test the hypothesis, a proposed experiment has been suggested and outlined. In the proposed experiment, a variety of indicator species as well as CL2 designated pathogens would be tested for at regular intervals throughout the cadavers' lifespans.

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List of Abbreviations

API	Analytical Profile Index
ATP	Adenosine trisphosphate
CL1	Containment Level 1
CL2	Containment Level 2
EHS	Environmental Health and Safety
HIV	Human Immunodeficiency Virus
GIDRU	Gastrointestinal Disease Research Unit
ICP-SP	Imperial College London soft-preservation
KGH	Kingston General Hospital
MERS	Middle Eastern Respiratory Syndrome
MSA	Mannitol Salt Agar
RG1	Risk Group 1
RG2	Risk Group 2

Chapter 1:

Introduction

1.1 History

The use of the human cadavers to teach anatomical sciences has been in use for centuries, with the earliest recorded dissection taking place in ancient Greece during the 3rd century BCE at the school of Greek medicine in Alexandria [1]. This form of active dissection soon disappeared from records due to changing religious attitudes whereby it was then considered to be blasphemous and against the will of the Church. The revival of dissection to teach anatomy began in the 14th century CE Italy and has continued ever since; it was then that prominent anatomists soon began expanding on the practice, including major changes in pedagogy with the practice of dissection being performed by the anatomists themselves as opposed to the barber surgeon, a move triggered by Andreas Vesalius [2]. In the present day, active dissection is a major component in teaching of the anatomical sciences, with the practice heavily valued by both graduate students (MSc, Medical Residents) and undergraduate students (BSc, Medical students) [3] [4]. The use of cadavers, however, is not without its own set of hazards—infectious risk is of primary concern, with the body acting as a reservoir for both normal flora and pathogenic microbes after death; hence use of chemicals to both preserve and disinfect the cadaver, a process known as embalming.

The act of embalming has been practiced for millennia—preceding the earliest recorded use of human cadavers for teaching—originating more than 3,000 years ago in ancient Egypt [5]. This embalming process, commonly known as mummification, was used by both the Chinchorro culture of modern-day Chile as well as the ancient Egyptians (c. 7020 and 3500 BCE, respectively). Egyptian culture believed that mummification prevented bodily decay thereby allowing for eternal life; on the contrary, those whose body decayed would be excluded from the afterlife [5]. It is these historical practices which modern embalming originated from—beginning with the use of arsenic and phenol-based solutions until the advent of formaldehyde by the German chemist August Wilhelm von Hofmann in the mid 19^{th} century [5]. Since then, very little has changed in the basic chemistry or technique of formaldehyde-based preservation of human cadavers.

1.2 The Process of Death

The post-mortem period is a familiar term described as the period after death, during which a predictable set of changes is seen to occur in the body. While, in this context, one assumes death to occur at a single point in time, this may not be the most appropriate way to conceptualize it; after all, cells are seen to continue to maintain some function even after the cessation of cardiovascular activity [6]. Death can perhaps more appropriately be thought of as a process in which a continuum of changes is seen in the body, ultimately leading to the total cessation of all biological functions vital to the survival of an organism. Due to its linear aspect of progression, it can be easiest to describe the process of death based on changes in order of appearance: immediate changes, early changes, and late changes.

Immediate changes in the process of death relate to somatic (clinical) death. Somatic death refers to the cessation of vital functions such as respiration and circulation; these can be checked most easily by means of a stethoscope in which the stethoscope is placed over the lungs and heart. It is at this stage where clinical death is often announced.

Early changes in the process of death are related to cellular death. With the cessation of vital functions, the body on the cellular level is no longer supplied with the necessary heat, nutrients, oxygen, and waste removal that is needed to sustain cellular life. As a result of this, basic cellular processes necessary for cell survival such as the production of adenosine trisphosphate (ATP) begin to halt. ATP is required for muscle relaxation and its absence results in the failure of muscles to relax, resulting in extreme rigidity and hardness of the muscles, a state known as 'rigor mortis'. With the cessation of circulation, heat is no longer transferred inside the body; this, combined with the onset of cellular death and resulting lack of heat production, leads to a decrease in body temperature, a process known as 'algor mortis'. Additionally, loss of circulation leads to blood pooling in body tissues of lower elevation, leading to post-mortem staining of these regions, a process known as 'livor mortis'.

Late changes arise and typically involve the breakdown of cells and tissues and growth of microbial life. Autolysis is a process by which cells and tissues begin to breakdown as a result of the release of intrinsic cellular enzymes responsible for digestion. Upon death, cells lose their ability to maintain homeostatic balance of the intracellular environment—cell membranes break down causing release of autolytic enzymes (i.e. those found within the lysosome and peroxisome), thereby resulting in autolysis. Moreover, as the body's physiology begins to deteriorate, so does its ability to defend against both pathogens and commensal microbes, allowing these microbes to grow unchecked as they consume the proteins and carbohydrates of the body [7]. Through a process called putrefaction, these pathogens and commensal microbes cause the gradual decomposition of the body.

Putrefaction has various signs which include discolouration, odour, gas production, and purge. The first sign of putrefaction is often a greenish discolouration of the skin over the right iliac fossa, the region overlaying the cecum, spreading across the abdomen and then to the head, neck, shoulders, and limbs. The cause of this discolouration is the reaction of hydrogen sulfide—a compound with a foul odour of rotten eggs and a by-product of anaerobic sulfate-reducing bacteria—with hemoglobin, forming the compound known as sulphahemoglobin [8]. As bacterial activity increases, the accumulation of gases occurs causing bloating, swelling, and expulsion of bodily gases, liquids, and semisolids from orifices—a process known as purging [8].

1.3 Post-Mortem Microbial Activity

Conceptually, one can recognize that the integrity of barriers of the innate immune system (i.e. mucosal surfaces), as well as the overall functioning of the immune system (i.e. innate immune system phagocytes, adaptive immune system lymphocytes, and etc.) will decline post-mortem; providing the opportunity for microbial spread. This process is the basis for the four ways in which positive bacterial samples occur in autopsy samples: true positive, agonal spread, post-mortem migration, and contamination [9].

True positive bacterial samples represent bacteria which were present in life and have continued to colonize, perhaps to a greater extent, after death. Typically, true positive samples will be pure growth of a single pathogen or normal floral species [10]. This is in contrast to the other three routes of bacterial colonization, which vary in time of infection during the process of death and typically yield mixed growth samples. In agonal spread, colonization occurs from bacterial invasion—typically from the gut microenvironment—during the process of death due to decreased mucosal integrity (i.e. during the death struggle or during resuscitation when circulation is somewhat maintained). In post-mortem migration, colonization occurs akin to agonal spread but after circulation has ceased and putrefaction has begun. Finally, contamination occurs via bacterial invasion from external sources, such as from sampling techniques or unsterile handling of the body [10].

While bacteria comprise a large portion of microbial activity post-mortem, they are not the only microorganism to colonize the dead. A variety of fungal and eukaryotic microbial taxa have also been shown to colonize human skin during decomposition [11]. Viral infectious agents and prions are also not to be excluded; diseases and organisms which may pose particular risks in cadavers include Creutzfeldt-Jakob disease, viral hepatitis, human immunodeficiency virus (HIV), Middle East Respiratory Syndrome (MERS) virus, and viral hemorrhagic fevers (Lassa, Ebola) [12].

Within the contexts of anatomical education, the goal of the embalming process is to minimize each opportunity for microbial colonization so that the embalmed cadaver possesses maximum sterility: cause of death and a brief medical history (i.e. screening for HIV infection) for eligibility in a Human Body Donor program minimizes infectious risk; promptly acquiring and properly storing bodies for embalming minimizes time for putrefaction and post-mortem migration; utilizing embalming materials which disinfect the tissue eliminates native flora and pathogens; utilizing fixatives (preservatives) in the embalming solution prevents further contamination by preventing microbial nourishment.

1.4 Embalming Process

While the components and materials used for embalming has changed over time, the desired end product has not changed since the dawn of embalming by the Chinchorro culture and ancient Egyptians: a preserved, sterile, and life-like body. Primary goals of embalming are fixation and sterilization. In lay terms, fixation refers to the act of creating a cadaver relatively immune to decomposition, and sterilization refers to freeing a cadaver of microbial life. Decomposition is primarily driven by microbial action, and thus sterilization aids in fixation. Secondary goals of embalming come after primary goals have been achieved and focus on the preservation of life-like characteristics including shape, colour, texture, flexibility, elasticity, and resistance [13]. Secondary goals are often strongly sought after when the cadaver is to be used for surgical training, with normal fixation and sterilization sometimes being sacrificed in order to maintain the life-like feel and appearance. This, however, poses a biological hazard for those working with the tissue and warrants increased biosafety precautions needed for handling.

Today, embalming is typically performed by means of fluid injection into the body. An arterial tube, also known as a cannula, is inserted into a major artery. The arterial blood is then allowed to drain from the body and is replaced with the embalming solution. Venous blood is often not removed as once coagulated it helps with identification of the veins. The injection of embalming fluids is achieved via a gravity feed or centrifugal pump. The solution is allowed to perfuse the tissues of the body for a few days and needle injection of specific non-perfused regions occurs. When the embalming process is complete, the body is said to be fixed. To ensure minimal decomposition and complete fixation, the cadaver is kept refrigerated throughout the process and for up to one-year post-embalming.

1.5 Embalming Chemistry

Embalming solutions are composed of chemical constituents, each of which having a specific purpose in meeting the primary and secondary goals of embalming: a preservative to fix the tissue, a disinfectant to sanitize, a humectant to maintain moisture, and a vehicle to dilute the mixture [14]. These basic components can be complimented with other ingredients such as dyes to retain colour and anticoagulants to break up blood clots, depending on individual needs.

Formaldehyde (CH_2O) is a colourless and pungent gas which is dissolved in water with other ingredients (i.e. methanol), a solution known as formalin. The main function of formaldehyde is to preserve biological specimens via the cross-linking of tissue proteins, effectively "locking in" their structure and preventing further functioning [15]. In this way, formaldehyde functions as a preservative to fix the tissue and is one of the most common preservatives used in embalming today. This molecular cross-linking resulting in inactivation of protein also acts on the microbial population; thus, formaldehyde has a secondary function as a disinfectant. The molecular cross-linking caused by formaldehyde hardens the tissue, preventing autolysis and accessibility to microorganisms, thereby preventing its decay [16] [15]. Despite its widespread use, formaldehyde is not without its own set of limitations. It is now agreed that formaldehyde acts as a carcinogen and appropriate measures should be taken to reduce exposure [17]; it discolours tissue by giving them a grayish hue; finally, it greatly stiffens muscles and decreases flexibility of joints which thought to be a result of actin-myosin linkage fixation [18] [19] [20].

In addition to the secondary disinfecting properties of formaldehyde, other chemicals are often added to the solution to aid in the sterilization process during embalming. Phenol or carbolic acid (C_6H_5OH), along with phenolic compounds, cause denaturing and inactivation of proteins/enzymes and ruptures lipid-based plasma membranes [21]. Its primary function is to act as an antiseptic and disinfectant as a result of good tissue penetration power combined with strongly bactericidal/fungicidal properties when used at concentrations of 1–2% [22]. A secondary function of phenol is to moderate tissue fixation—it has proven to show considerable promise in preserving tissue in a life-like state as shown through the maintenance of native biomechanical properties while mitigating infectious risk [23]. The brand name compound Dettol (C_8H_9ClO) is another phenolic compound which functions as an antiseptic and disinfectant by causing denaturing and inactivation of proteins/enzymes, and rupturing of lipid-based plasma membranes. It often is incorporated into embalming solutions as an additional method of disinfection.

A common result of formaldehyde fixation and the embalming process in general is tissue dehydration, resulting in a loss of form and shape. As such, hygroscopic humectants are commonly utilized in order to counteract this tissue dehydration by restoring cellular hydration. Common humectants include glycerol (also known as glycerine), sorbitol, ethylene glycol, and propylene glycol.

In order to properly distribute the mixture, it must first be diluted using a vehicle or solvent to form a solution. A common vehicle which is utilized is ethanol due to its antimicrobial properties. Its mechanism of action is similar to that of phenol whereby it causes protein denaturation and lipid dissolution [21]. Water is also a common vehicle that is used in embalming solutions.

1.6 Embalming Solutions

Various solutions exist today which can be used in the embalming process. The ideal solution is one which minimizes the infectious risk associated with handling the cadaver, minimizes exposure to harmful/carcinogenic substances when handling the cadaver (i.e. formaldehyde), and maximizes the preservation in a natural life-like state of the cadaver. In achieving this ideal solution, a delicate balancing act must occur between these three desired qualities; for example, a solution with a high formaldehyde concentration will undoubtedly sterilize the cadaver and prevent microbial colonization by fixation, but it will also result in a cadaver which is discoloured, stiff/rigid, firm, and morphed in shape.

Formalin-based embalming results in a cadaver that is sterile, long lasting, and well fixed. This traditional, primarily formalin-based embalming method is commonly referred to as "hard-fixed" [24]. Common components of this type of solution include solvents such as water and/or ethanol, humectants such as glycerine and/or propylene glycol, disinfectants such as Dettol and/or phenol, and finally a relatively high concentration of formaldehyde. A major drawback to this embalming method is a loss of the natural life-like state of the cadaver.

Major alternatives to the traditional formalin-based "hard-fixed" embalming solution are known as "soft-fixed" solutions due to their ability to retain a more natural state of the cadaver [24]. These solutions can contain small amounts of formaldehyde or no formaldehyde at all, however no cadaver which is embalmed using one of them can be described as hard-fixed. The Thiel method was developed by Walter Thiel in 1992 and has since been in use in many facilities due to its ability to produce cadavers with well-preserved texture and colour [25] [26]. The method involves fully submerging the cadaver in a tank of embalming fluid consisting of a small percentage of formaldehyde along with salts such as ammonium nitrate, potassium nitrate, and sodium sulfate. Other soft-fixed cadavers make use of the ability of phenol to act as both a disinfectant and partial preservative and thus substitute for formaldehyde. These phenol-based solutions have also been well regarded due to their ability to produce a natural life-like cadaver and have been implicated for use in postgraduate surgical training [27]. The solution typically consists of ethanol, water, glycerol, and phenol [27].

1.7 Laboratory Safety

In Canada as in many parts of the world, an extensive and enforced set of guidelines exist for maintaining biosafety in facilities which handle and store human pathogens or toxins, including anatomical teaching and research laboratories [28]. In this context, biosafety is defined as a set of practices which minimize or prevent harm to facility workers, the public, and the environment. All anatomical dissection and teaching laboratories must develop and receive approval on a properly-constructed biosafety program; such a program includes information such as biological material and hazards present in the laboratory, risk assessments of pathogens, and containment level zones.

Biological material refers to any micro-organism (microbe), protein, or nucleic acid, including those materials which contains them such as body tissue. Pathogens are biological material which have the potential to cause disease. Humans are naturally colonized by non-pathogenic microbes—bacteria, viruses, and fungi—and collectively, these micro-organisms are known as the normal microflora. "Hotspots" for microbial life in the human body include the nasal region, oral region, surface skin, gastrointestinal tract, and urogenital tract/region. Estimates indicate that the adult human is covered with approximately 2 m^2 of skin which supports approximately 10^{12} bacteria alone; additionally, approximately 10^{14} bacteria exist in the gastrointestinal tract [29]. This normal microflora plays important roles in normal human functioning such as preventing infection and providing nutrients. Some normal microflora has the potential to cause disease if given the opportunity (i.e. decreased competition with other species, change in environmental conditions, disruption in host's immune system or barriers), and these micro-organisms are called opportunistic pathogens. Some examples of the normal microflora include: *Candida albicans*, a fungus normally found in the gastro-intestinal tract; *Staphylococcus aureus*, a potential bacterial pathogen which is also part of the normal microflora of the nares; and bacterial *Escherichia coli*, found in feces.

Risk is a measure of the probably of an unlikely and undesired event occurring. A risk assessment is an evaluation of the risk associated with a particular action, pathogen, or toxin. These assessments are completed by a variety of individuals with varying expertise and take into consideration a variety of risk factors including pathogenicity (ability to cause disease), route of infection, infectious dose, and impact of release into the environment. Upon completion, pathogen risk assessments determine to which Risk Group the pathogen belongs to. Risk Groups are scaled on a range of 1–4 with Risk Group 1 (RG1) signifying the lowest individual and community risk and Risk Group 4 (RG4) signifying the highest individual and community risk [28]. Risk Group 2 pathogens pose a moderate risk to the individual and low risk to public health and include pathogens such as *Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Staphylococcus aureus, Candida albicans,* and Hepatitis B, C, and E viruses [30].

Containment levels describe the set of minimum physical containment and operational practices necessary for work in that area. In Canada, there are 4 containment levels ranging from Containment Level 1 (CL1) for work with Risk Group 1 materials to Containment Level 4 (CL4) for work with Risk Group 4 materials. CL1 facilities do not have any specific physical or operational requirements above and beyond basic design elements such as handwashing sinks and good microbiological laboratory practices [28]. Hard-fixed cadavers and tissue are typically designated as a RG1 material and are therefore accessible in CL1 facilities which are widespread at colleges, universities, and teaching hospitals. CL2 facilities build on the basic design elements found in CL1 facilities by incorporating additional operational practices and physical containment requirements proportional on the risk associated with the materials handled within [28]. Operational practices which are employed in CL2 include mandatory biosafety training, active biosafety program management, standard operating procedures, personal protective equipment, and decontamination protocols. Physical containment requirements which are employed in CL2 include facility layout specifications (i.e., handwashing sinks next to exits, locked doors separating public areas from containment zones), restricted access, specific surface finishes (i.e., stainless steel), and biological safety cabinets. Due to the paucity of data surrounding microbial activity in soft-fixed cadavers, they are typically designated as a RG2 material and are therefore only accessible in CL2 facilities. This poses a challenge for those looking to gain valuable insight from work with these life-like materials as CL2 facilities are not as common due to their higher cost of construction and operation.

Chapter 2:

<u>The Hypothesis</u>

Current EHS restrictions place traditional formalin-based hard-embalmed cadavers in CL1 laboratories, with both fresh/frozen and phenol based soft-embalmed cadavers in CL2 laboratories. However, there is a gap in the current literature dictating whether or not phenol based soft-embalmed cadavers do in fact pose significant biological hazard to warrant restriction to CL2 laboratories. While CL2 facilities ensure greater protection than CL1 facilities against potential biological hazards, they are both more costly to build and operate. As a result, many institutions and students are unable to gain access to such facilities, hindering their potential to learn from the specimens requiring clearance. For example: at Queen's University, access to life-like soft-embalmed tissue in the anatomy CL2 laboratories is only given to graduate students (MSc, PhD, and Medical Residents) and withheld from undergraduate students (BSc, Medical students). Given the life-like qualities of phenol-based soft-embalmed cadavers, the potential for having these cadaveric specimens in CL1 facilities poses significant opportunity for students in anatomical education. Herein forms the rationale for the generation of this thesis and the inclusion of the proposed experiment.

For the purposes of this thesis, it was hypothesized that soft-embalmed cadavers are a higher risk biosafety hazard than formalin-embalmed cadavers. Accordingly, it was predicted that soft-embalmed cadavers will possess both a greater variety and quantity of pathogenic microbes than formalin-embalmed cadavers. This hypothesis and prediction, if supported by results from the evaluation and proposed experiment, would indicate that phenol based soft-embalmed cadavers are justly designated to CL2 facilities.

Chapter 3:

Evaluation of the Hypothesis

Considering the age of formalin-based hard-embalming, which originated in the mid 19th century [5], it would not be outlandish to assume that many institutes of anatomy across the world are aware of it. In fact, in a recent study conducted by Benkhadra *et al*. (2011), formalin-based solutions were found to be the most commonly used embalming solutions worldwide [25]. This is especially interesting for a number of reasons: the per-unit cost of formalin-based solutions exceeds that of some alternatives; formalin-based solutions do a relatively poor job at preserving the natural life-like properties of cadaveric tissue including range of motion, colour, and texture; and formaldehyde is generally considered to be carcinogenic [17] [20] [31]. Indeed, however, the positives of formalin-based embalming are clear: it does not require complex equipment; the solution recipe is relatively simple; the completed cadavers can be worked on in low containment level facilities; sterility of the tissue is maintained; and fixation of the tissue is maximal allowing for accurate dissection, akin to a sculpture [20] [25] [31]. The ideal solution will be one which satisfies all or close to all of the aforementioned points.

Of the numerous types of soft-embalming techniques in use today, the Thiel method [26] is considered by some to be one of the best [32]. The Thiel method makes use of large amounts of salts for fixation, phenolic compounds and boric acid for disinfection, ethylene glycol as a humectant, and small amounts of formalin for fixation and disinfection [26]. In this method, the cadavers are perfused with an intravascular solution via the femoral or carotid artery and then are submerged in an immersion solution for approximately 6 months, resulting in a cadaver which can be used for months or years [26]. The resulting cadaver is praised for its almost natural colours [25], haptic properties [25] [33], and strong sterility [26]. Its use in various medical disciplines (i.e. surgical skills training) is well documented [25] [33]. It is, however, not without its own set of limitations. In 2014, Hammer *et al*. reported major histological flaws associated with sections taken from Thiel embalmed tissue as a result of harsh fixatives present, such as ruptured or missing cell membranes and nuclei resulting in blurred structural borders [33]. Additionally, it has been noted that the Thiel method is quite costly—both in creating the solution and financing the necessary equipment (i.e. full body submersion tanks) [33]. Disintegration of muscular tissue using the method has also been documented [5]. In a paper by Hammer *et al*. (2014), a modified Thiel method was introduced which was hypothesized to act as a better alternative for the original Thiel method [31]. However, Thiel fixation is still noted as more expensive, hazardous, odorous, toxic, prone to deterioration and less suitable for histological testing than other soft-embalmed techniques [33]. Additionally, similar to other tested soft embalmed techniques by Hammer *et al*. in 2014, biomechanical properties are still seen to be irreversibly altered [33].

In 1998, Coleman and Kogan developed an additional soft-embalming technique known as the Saturated Salt Solution. This solution was characterized by high levels of common salt (NaCl), which is readily available and cheap, combined with low formaldehyde content [13]. It was argued that the high salt content of this new embalming solution would create a cadaver which was resistant to significant desiccation. No investigation was done on this new proposed embalming solution until 2014, when Hayashi *et al*. studied it in detail for surgical skills training against other commonly used embalming methods [31]. The Saturated Salt Solution was found to have advantages against the Thiel method in natural colour retention, relatively low deterioration, imaging and histological quality, and solution cost [31] [32] [34] [20]. Still, however, disadvantages of the embalming method have been noted: somewhat rigid joints and tissues, expensive equipment needed (similar to Thiel method, cadavers are kept in submersion vessels for at least 3 months [13]), and edematous nature [31] [32]. In addition, the infectious risk of traditional formalin hard-embalmed cadavers appears unmatched [32] [35]. It can be noted however that some recent reports have found superior results in range of motion for saturated salt solution embalmed cadavers used in surgical skills training when compared to both formalin hard-embalmed and some soft-embalmed techniques [20].

The search for soft-embalming techniques which result in comparable infectious risk to the traditional formalin hard-embalming technique is one which surprisingly has not garnered significant attention in academia. Only a few studies have looked at the disinfecting efficiency of various embalming fluids in recent times, with only 1 assessing soft-embalming techniques [31] [35] [36] [37]. Furthermore, 2 recent studies reported contradicting information regarding the sterility of formalin hard-embalmed cadavers [31] [36]. This poses an obstacle for gathering enough evidence to definitively judge proper containment level designation for cadavers in anatomy laboratories. Results from a recent study done by Balta *et al*. (2018) showed that soft-embalmed cadavers contained varying amounts of micro-organisms relative to hard-embalmed cadavers depending on body region sampled, with some regions demonstrating more colony forming units of micro-organisms when embalmed using soft-embalming versus hard-embalming solutions [35]. However, no known comparative identification analysis has been completed to date on the types of micro-organisms present in cadaveric remains embalmed using hard- versus soft-embalming techniques. It is also not known what micro-organisms, pathogenic or not, are killed by the soft-embalming solutions studied by Balta *et al*. (2018). One of the soft-embalming solutions in use in this study, Imperial College London soft-preservation (ICP-SP), closely resembles the soft-embalming solution used in the proposed experiment of this report; containing ingredients of alcohol, water, glycerol, and phenol [24]. In this study, ICP-SP stayed bacterial colony free until the 4th month, reaching full colony growth in by the 6th month in one cadaver [24]. This is in contrast to Thiel and formalin hard-embalmed cadavers, which saw little to none bacterial colonies over the 8-month study duration [24]. These data suggest that phenol based soft-embalming solutions effectively sterilize the cadaver for a short duration but are unable to maintain total sterilization for as long as formalin hard-embalmed or Thiel soft-embalmed cadavers. What is unknown, still, is what types of bacteria and micro-organisms are seen to colonize these cadavers—pathogenicity of these micro-organisms as it relates to laboratory biosafety cannot be determined without partial identification.

Few studies have looked at the identification of microbial contents of embalmed cadavers. One study, done by Tabaac *et al*. in 2013, tested for various bacterial species in formalin-embalmed cadavers [36]. After embalming, sterile swabs collected from the axilla, oronasal, and perineal regions were used to inoculate 4 different culture media: 5% sheep blood Baird-Parker Agar (BPA) plates for isolation of *Staphylococci* [38], 5% sheep blood Phenylethyl Alcohol Agar plates for isolation of gram-positive cocci [39], Mannitol Salt Agar (MSA) plates for isolation of *Staphylococci* [40], and nutrient agar plates. Sheep blood agar can be used as a primary plating medium which displays hemolysis as a result of bacterial growth [41]. The plates were cultured, and morphological characteristics of the isolated individual colonies recorded. Slides were also generated from the colonies and gram stained to confirm purity and ensure morphology. Confirmed pure samples were subject to both the catalase test to determine catalase-positive species [42], and the coagulase test to determine coagulase-positive *Staphylococci* [43]. Analytical Profile Index (API) strips are a manual method of microorganism identification based on a series of chemical reactions [44]. API strips were selected according to the previously acquired sample data and then inoculated with the respective samples. Final sample identification was done using the information gathered from the API test provided there was agreement with the previously recorded sample data. Results from this study showed a variety of bacterial species present from each formalin-embalmed cadaver and each sample location [36]. This, however, contradicts the findings of Hayashi *et al*., 2014 [31] and Balta *et al*., 2018 [35]. A possible explanation for this could be contamination of the samples, or the sample collection time post-embalming which was not stated. Balta *et al*. (2018) found that soft-embalmed cadavers had minimal to no colonization immediately post-embalming, continuing for a few months until colonization picked up; this is likely due to post-mortem spread or contamination [35].

With regards to laboratory safety, it is unknown which microorganisms were present on the soft-embalmed cadavers demonstrated by Balta *et al*., 2018 [35]. Specifically, it is not known whether or not microorganisms which are RG2 designated would be found on these cadavers, thus justifying their rightful restriction to CL2 facilities. In this report, I propose an experiment to help elucidate these unanswered questions.

Chapter 4:

The Proposed Experiment

Through an in-depth analysis of the literature, evidence for soft-embalmed cadavers harbouring more microbial life than formalin hard-embalmed cadavers has been found. To definitively test the hypothesis, an experiment was proposed to elucidate the risk associated with soft-embalmed cadaver microbial life. Both the materials and a detailed list of protocols for the proposed experiment can be found in this chapter.

4.1 Materials

A. The Embalming Process

   a. Designate cadavers for formalin-based hard-fixation
   b. Designate cadavers for phenol-based soft-fixation
   c. Stainless steel cadaver tables
   d. Route for disposal of bodily remains or body-related materials (i.e. autoclave, incinerator)
   e. Body bags
   f. Freezer for body storage pre-embalming
   g. Dettol disinfectant at 1–3% concentration
   h. Surgical tools for access to vasculature (i.e. scalpel, haemostat, tweezers, probe, scissors)
   i. Embalming machine with embalming tubes and attached cannulas
   j. Cotton wrap

k. Moistening solution
    i. 250mL Dettol
    ii. 300g Potassium Acetate
    iii. 1500mL Glycerine or Propylene
    iv. Glycol
    v. Distilled water is added to make up to a 22L solution
l. Soft-embalming solution
    i. Phenol
    ii. Ethanol
    iii. Water
    iv. Glycerin
m. Hard-embalming solution
    i. 500mL Potassium Acetate (saturated solution in H_2O)
    ii. 600mL Dettol
    iii. 500mL Phenol (Carbolic Acid)
    iv. 500mL Formalin (40% Formaldehyde)
    v. 1000mL Propylene glycol (or glycerine)
    vi. 95% ethanol (unmatured) is added to make up to a 22L solution

B. Sample Collection and Transport
    a. Sterile sample swabs
    b. Labelled, double contained, surface disinfected transport vessels as per Queen's University biosafety regulations on movement of pathogens or microorganisms.

C. Testing

a. Analytical Profile Index (API) strips [44]

b. Baird-Parker agar (BPA) plates (identification of *Staphylococci*) [38]

c. Phenylethyl alcohol agar plates (identification of gram-positive cocci) [39]

d. Mannitol salt agar (MSA) plates (identification of *Staphylococci*) [40]

e. Catalase and coagulase tests (Remel Staphaurex Plus, ThermoFisher Scientific) (identification of *Staphylococcus aureus*) [42] [43]

f. Tryptic Soy agar (TSA) with yeast extract plates (identification of *Listeria monocytogenes*) [45]

g. Xylose-lysine-deoxycholate agar (XLD) plates (identification of gram-negative bacilli—*Salmonella typhimurium, Shigella flexneri*) [46]

h. Cetrimide agar plates (identification of *Pseudomonas aeruginosa*) [47]

i. Sabouraud agar plates (identification of fungi) [48]

j. Nutrient agar plates (for non-pathogenic microbes)

k. Inoculation loop

l. Bunsen burner

m. Incubator

4.2 Methods

A. Arrival of Cadavers

1. Cadavers will be delivered wrapped or covered in a shroud and/or plastic or cotton sheet. They may or may not have documentation regarding any known communicable pathogens or other recognized hazards accompanying them.

2. If proper documentation is received (i.e. cause of death and/or certification that no known communicable pathogens or other recognized hazards are present) then embalming can proceed.
3. After unwrapping the cadaver, the sheet or shroud and any other accompanying materials will be autoclaved and disposed of by incineration.
4. The cadaver will be placed in a plastic body bag and stored in a freezer bank.

B. Preparation for Storage

1. The cadaver will be removed from the freezer bank.
2. The cadaver will be bathed in a Dettol disinfectant, a highly effective germicide for cadavers when used at 1–3% concentration.
3. Any medical devices (i.e. colostomy tube, IV tube, etc.) will be removed and autoclaved prior to disposal.
4. Surface hair will be shaved from the body.
5. The cadaver will be assigned a metal cadaver table for future use. The age, sex, and cause of death of the donor will be displayed on the table.

C. Embalming Cadavers

1. Joints will be manipulated to increase flexibility and prevent arterial constriction
2. Incisions will be made into the femoral arteries, thus allowing drainage of arterial blood. Venous blood will not be removed unless the venous system is going to be used for special dissections. Once coagulated, the venous blood helps with identification of the veins.
3. Embalming tubes will be inserted into the femoral arteries and secured with ligatures. Two tubes will be inserted into the left femoral artery, one up and one

down. Another tube will be inserted into the right femoral artery and the ascending portion of this right femoral artery tied off.

4. The cadaver will be injected with the embalming fluid. Injection pressure should be maintained at approximately 8-10 psi. Embalming fluid will be injected until the area being infused appears firm and contour hollows are slightly filled.
5. When embalming is complete, the embalming tubes should be immediately removed and cleaned with cold running tap water. The arteries will be tied off and the sites of injection sutured.
6. Following embalming, the cadaver will be left for a period of 1-2 days to allow the embalming fluid to diffuse through arterial walls and into the surrounding tissues. Needle injection of specific regions can be performed as required.

D. Storage of Anatomical Materials

1. Before storing, the head, hands, and feet will be wrapped in toweling and covered with an outer cotton wrap. This will then be moistened with moistening solution.
2. A plastic bag will be placed around the head, hands, and feet.
3. To assure complete fixation of all tissues and to prevent bacterial decomposition, cadavers will be kept refrigerated at 4°C in the morgue area for a period of:
    i. 8–12 months for formalin-embalmed cadavers.
    ii. 1.5 weeks for soft phenol-embalmed cadavers.

E. Sampling of Anatomical Materials

1. A sterile sample swab will used to sample the microflora of the cadaver at four locations:
    i. Oral cavity

ii. External nares (nostrils)

iii. Rectum

iv. Axilla (armpit)

2. A total of 4–5 samples will be collected per location for each cadaver: pre-embalming, 1-month post embalming, 2-months post embalming, 3-months post embalming, and tentatively 4-months post embalming.

F. Transport of Anatomical Materials

1. Collected sample swabs will be placed in double contained transport vessels.
2. The transport vessels will be surface disinfected and labeled.
3. A trusted member of the research team will transport the samples to the Gastrointestinal Disease Research Unit (GIDRU) at Kingston General Hospital (KGH) on site of Queen's University.

G. Testing of Indicator Species and Pathogenic Microbes

1. Following recommended procedure for each agar and API strip, the plates and the appropriate API strips will be inoculated via the collected sample swabs.
2. Plates and slips will be incubated according to recommended procedure.
3. Catalase and coagulase tests will be completed according to recommended procedure.
4. The plates will be examined for colony growth.
5. Plate colony growth will be cross referenced with collected API strip and catalase/coagulase test data.

Chapter 5:

Consequences of the Hypothesis and Discussion

After an in-depth analysis of the present literature surrounding the use and properties of soft-embalmed cadavers in comparison with traditional formalin hard-embalmed cadavers, it was found that soft-embalmed cadavers do become colonized more readily by micro-organisms, aligning with our hypothesis which states that soft-embalmed cadavers are a higher risk biosafety hazard than formalin-embalmed cadavers. Biosafety risk is not necessarily increased, however, simply because more microbial life is present on the cadavers. This is evidenced by the vast quantity of microbial life making up the natural human microbiome, such as in the gastrointestinal tract [49]. To more accurately estimate biosafety risk of soft-embalmed cadavers, the pathogenicity of the micro-organisms present must be determined. Thus, to definitively test the hypothesis, a proposed experiment has been suggested and outlined. In the proposed experiment, a variety of indicator species as well as RG2 pathogens would be tested for at regular intervals throughout the cadavers' lifespans.

While CL2 facilities ensure greater protection than CL1 facilities against potential biological hazards, they are both more costly to build and operate. The consequences of our hypothesis are two-fold: from a health and safety standpoint, we will be more confident in our actions to restrict soft-embalmed cadavers to CL2 facilities, providing informed due protection to employees and students; from a financial and operational standpoint, we will be more content with our investments into the building and operation of CL2 facilities, however less institutions and students will be able to benefit from this learning tool within the context of anatomical education.

Literature Cited

[1] I. Serageldin, "Ancient Alexandria and the dawn of medical science," *Global Cardiology Science & Practice,* pp. 395-404, 2013.

[2] M. E. Silverman, "Andreas Vesalius and De Humani Corporis Fabrica," *Clinical Cardiology,* pp. 276-279, 1991.

[3] A. J. Turner, A. Mellington and F. Ali, "Fresh cadaver dissection for training in plastic surgery," Elsevier, East Grindstead, 2005.

[4] D. J. Jones, "Anatomy Departments and Anatomy Education: Reflections and Myths," *Clinical Anatomy,* pp. 34-40, 1997.

[5] E. Brenner, "Human pody preservation—old and new techniques," *Journal of Anatomy,* pp. 316-344, 2014.

[6] Vrselja, Z., Daniele, S.G., Silereis, J, Talpo, F, Morozov, Y, Sousa, A, Tanaka, B, Skarica, M, Pletikos, M, Kaur, N, Zhuang, Z, Liu, Z, Alkawadri, R, Sinusas, A, Latham, S, S. Waxman and N. Sestan, "Restoration of brain circulation and cellular functions hours post-mortem," *Nature,* pp. 336-343, 2019.

[7] R. Shedge, K. Krishan, V. Warrier and T. Kanchan, "Postmortem Changes," in *StatPearls*, Treasure Island (FL), StatPearls Publishing, 2020.

[8] V. Lynch, Forensic Nursing, St. Louis (MO): Elsevier Mosby, 2006.

[9] J. A. Morris, L. M. Harrison and S. M. Partridge, "Postmortem bacteriology: a re-evaluation," *Journal of Clinical Pathology,* vol. 59, pp. 1-9, 2006.

[10] S. Tuomisto, P. Karhunan, R. Vuento, J. Aittoniemi and T. Pessi, "Evaluation of Postmortem Bacterial Migration Using Culturing and Real-Time Quantitative PCR," *Journal of Forensic Sciences,* vol. 58, pp. 910-916, 2013.

[11] J. Metcalf, Xu, Z, Weiss, S, Lax, S, Treuren, W, Hyde, E, Song, SJ, Amir, A, Larsen, P, Sangwan, N, Haarmann, D, Humphrey, G, Ackermann, G, Thompson, L, Lauber, C, Bibat, A, Nicholas, C, Gebert, M, Petrosino, J, Reed, S, Gilbert, J, A. Lynne, and S. Bucheli, , "Microbial community assembly and metabolic function during mammalian corpse decomposition," *Science,* vol. 351, no. 6269, pp. 158-162, 2016.

[12] P. N. Hoffman and T. D. Healing, "Guide to Infection Control in the Healthcare Setting: The Infection Hazards of Human Cadavers," International Society for Infectious Diseases, 2018.

[13] R. Coleman and I. Kogan, "An improved low-formaldehyde embalming fluid to preserve cadavers for anatomy teaching.," *Journal of Anatomy,* vol. 192, pp. 443-446, 1998.

[14] L. R. Allen, "An Assessment of the Procedures Used for Anatomical Embalming within Canadian Tertiary Institutions," Queen's University at Kingston, Kingston, 2009.

[15] C. H. Fox, F. B. Johnson and P. P. Roller, "Formaldehyde Fixation," *Journal of Histochemistry and Cytochemistry,* pp. 845-853, 1985.

[16] J. T. Mason and T. J. O'Leary, "Effects of Formaldehyde Fixation on Protein Secondary Structure: A Calorimetric and Infrared Spectroscopic Investigation," *Journal of Histochemistry and Cytochemistry,* pp. 225-229, 1991.

[17] U.S. Department of Health and Human Services, "14th Report on Carcinogens," National Toxicology Program, Research Triangle Park, NC, 2016.

[18] C. Richins, E. Robers and J. Zeilmann, "Improved fluids for anatomical embalming and storage," *The Anatomical Record,* pp. 241-243, 1963.

[19] P. Vasicova, Rinnerthaler, M, Haskova, D, Novakova, L, Malcova, I, M. Breitenbach and J. Hasek. Formaldehyde fixation is detrimental to actin cables in glucose-depleted S. cerevisiae cells," *Microbial Cell,* pp. 206-214, 2016.

[20] D. Burns, Bell, I, Katchky, R, Dwyer, T, Toor, J, C. Whyne and O. Safir, "Saturated Salt Solution Cadaver-Embalming Method Improves Orthopaedic Surgical Skills Training," *Journal of Bone and Joint Surgery,* 2018.

[21] D. Demiryürek, A. Bayramoglu and S. Ustaçelebi, "Infective agents in fixed human cadavers: A brief review and suggested guidelines," *The Anatomical Record,* pp. 194-197, 2002.

[22] M. L. Wickstrom, "Phenols and Related Compounds," Merck Manual Veterinary Manual, October 2015. [Online]. Available: https://www.merckvetmanual.com/pharmacology/antiseptics-and-disinfectants/phenols-and-related-compounds. [Accessed May 2020].

[23] S. Lalonde, D. Pichora and S. Zakani, "Phenol Embalming: A New Model For The Biomechanical Study of Flexor Tendon Repair," *Orthopaedic Proceedings,* Vols. 98-B, 2018.

[24] J. Balta, M. Cronin, J. Cryan and S. O'Mahony, "Human Preservation Techniques in Anatomy: A 21st Century Medical Education Perspective," *Clinical Anatomy,* vol. 28, pp. 725-734, 2015.

[25] M. Benkhadra, Gérard, J, Genelot, D, Trouilloud, P, Girard, C, F. Anderhuber and G. Feigl, "Is Thiel’s embalming method widely known? A world survey about its use.," *Surgical and Radiological Anatomy,* vol. 33, pp. 359-363, 2011.

[26] W. Thiel, "[The preservation of the whole corpse with natural color]," *Annals of Anatomy,* vol. 174, pp. 185-195, 1992.

[27] D. P. Barton, Davies, D, Mahadevan, V, Dennis, L, Adib, T, Mudan, S, A. Sohaib and H. Ellis, "Dissection of soft-preserved cadavers in the training of gynaecological oncologists," *Gynaecological Oncologists,* vol. 113, pp. 352-356, 2009.

[28] "Canadian Biosafety Handbook, 2nd edition," Public Health Agency of Canada, Ottawa, 2016.

[29] R. Sender, S. Fuchs and R. Milo, "Revised Estimates for the Number of Human and Bacteria Cells in the Body," *PLoS Biology,* vol. 14, no. 8, 2016.

[30] "Schedule 2: Risk Group 2 Human Pathogens," in *Human Pathogens and Toxins Act*, Ottawa, Minister of Justice of Canada, 2019.

[31] S. Hayashi, Homma, H, Naito, M, Oda, J, Nishiyama, T, Kawamoto, A, Kawata, S, Sato, N, Fukuhara, T, Taguchi, H, Mashiko, K, Azuhata, T, Ito, M, Kawai, K, Suzuki, T, Nishizawa, Y, Araki, J, Matsuno, N, Shirai, T, Qu, N, Hatayama, N, Hirai, S, H. Fukui and M. Itoh, "Saturated Salt Solution Method: A Useful Cadaver Embalming for Surgical Skills Training," *Medicine,* vol. 93, pp. 1-10, 2014.

[32] S. Hayashi, Naito, M, Kawata, S, Qu, N, Hatayama, N, S. Hirai and M. Itoh, , "History and future of human cadaver preservation for surgical training: from formalin to saturated salt solution method," *Anatomical Science International,* vol. 91, pp. 1-7, 2016.

[33] N. Hammer, Löffler, S, Bechmann, I, Steinke, H, C. Hädrich and C. Feja, "Comparison of modified thiel embalming and ethanol-glycerin fixation in an anatomy environment: Potentials and limitations of two complementary techniques," *Anatomical Sciences Education,* vol. 8, pp. 74-85, 2014.

[34] M. Lombardero, Yllera, M, Costa-E-Silva, A, M. Oliveira and P. Ferreira , "Saturated salt solution: a further step to a formaldehyde-free embalming method for veterinary gross anatomy," *Journal of Anatomy,* vol. 231, pp. 309-317, 2017.

[35] J. Y. Balta, J. F. Cryan and S. M. O'Mahony, "The antimicrobial capacity of embalming solutions: a comparative study," *Journal of Applied Microbiology,* vol. 126, pp. 764-770, 2018.

[36] B. Tabaac, Goldberg, G, Alvarez, L, Amin, M, K. Shupe-Ricksecker and F. Gomez, "Bacteria detected on surfaces of formalin fixed anatomy cadavers," *Basic and Applied Anatomy,* vol. 118, pp. 1-5, 2013.

[37] P. Majewski, Pernak, A, Grzymislawski, M, K. Iwanik and J. Pernak; "Ionic liquids in embalming and tissue preservation. Can traditional formalin-fixation be replaced safely?," *Acta Histochemica,* vol. 105, pp. 135-142, 2003.

[38] ThermoFisher Scientific, "Baird-Parker Agar Base (Dehydrated)," [Online]. Available: https://www.thermofisher.com/order/catalog/product/CM0275B#/CM0275B. [Accessed September 2020].

[39] ThermoFisher Scientific, "BD BBL™ Dehydrated Culture Media: Phenylethyl Alcohol Agar," [Online]. Available: https://www.fishersci.ca/shop/products/bd-bbl-dehydrated-culture-media-phenylethyl-alcohol-agar/b11539. [Accessed September 2020].

[40] P. Shields and A. Tsang, "Mannitol salt agar plates protocols," American Society for Microbiology, [Online]. Available: https://www.asmscience.org/content/education/protocol/protocol.3034. [Accessed September 2020].

[41] Dalynn Biologicals, "Sheep Blood Agar," February 2003. [Online]. Available: https://www.dalynn.com/dyn/ck_assets/files/tech/PS58.pdf. [Accessed September 2020].

[42] K. Reiner, "Catalase Test Protocol," American Society for Microbiology, 2010.

[43] N. Chamberlain, "Coagulase test for staphylococcus species," American Society for Microbiology, 2009.

[44] BioMérieux Clinical Diagnostics, "API®," [Online]. Available: https://www.biomerieux-usa.com/clinical/api. [Accessed September 2020].

[45] Dalynn Biologicals, "Tryptic Soy Agar," [Online]. Available: https://www.dalynn.com/dyn/ck_assets/files/tech/PB81.pdf. [Accessed September 2020].

[46] Dalynn Biologicals, "XLD Agar," [Online]. Available: https://www.dalynn.com/dyn/ck_assets/files/tech/PX75.pdf. [Accessed September 2020].

[47] Dalynn Biologicals, "Cetrimide Agar," [Online]. Available: https://www.dalynn.com/dyn/ck_assets/files/tech/TC42.pdf. [Accessed September 2020].

[48] Dalynn Biologicals, "Sabouraud Dextrose Agar," [Online]. Available: https://www.dalynn.com/dyn/ck_assets/files/tech/TS24.pdf. [Accessed September 2020].

[49] P. Turnbaugh, Ley, R, Hamady, M, Fraser-Liggett, C, R. Knight and J. Gordon, "The Human Microbiome Project," *Nature,* vol. 449, pp. 804-810, 2007.