A HISTOLOGICAL ANALYSIS OF MAST CELLS IN THE BIOLOGICAL RESPONSE AROUND IMPLANTS

A DISSERTATION SUBMITTED

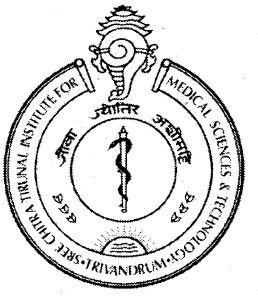
BY

SREELATHA.K.H

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY



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DECLARATION

I, **Sreelatha.K.H**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**A histological analysis of mast cells in the biological response around implants**" under the direct supervision of **Dr. Mira Mohanty, Scientist G, Division of Implant Biology,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**A histological analysis of mast cells in the biological response around implants**" submitted by **Sreelatha.K.H** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Histopathology Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ASTM	-	American Standards for Testing Materials
bFGF	-	basic Fibroblast Growth Factor.
COX 2	-	Cyclooxygenase 2
DAB	-	Diamino benzidine
FBGC	-	Foreign Body Giant Cells
FBR	-	Foreign Body Reaction
FDA	-	Food and Drug Administration
FGF	-	Fibroblast Growth Factor
H&E	-	Hematoxylin and Eosin
ICAM	-	Intercellular Cell Adhesion Molecule
IgG	-	Immunoglobulin G
IHC	-	Immunohistochemistry
IL	-	Interleukin
ISO	-	International Standards of Organizations
NIH	-	National Institute of Health.
PAR 2	-	Protease Activated Receptor 2
PBS	-	Phosphate Buffer Saline.
PDGF	-	Platelet derived Growth Factor
PGD2	-	Prostaglandin 2
PMMA	-	Polymethyl methacrylate
PTFE	-	Poly Tetra Fluro Ethylene
ROI	-	Reactive Oxygen Species
SCF	-	Stem Cell Factor
TJR	-	Total Joint Replacements
TNF	-	Tumor Necrosis Factor.
UHMWPE	-	Ultra High Molecular Weight Polyethylene
uM	-	Micrometer

SYNOPSIS

The biological response to an implanted biomaterial is a common defense mechanism by the body which may in some cases lead to rejection and the failure of the implants. The materials used in the manufacture of different medical implants are considered to be inert, but often has resulted in adverse tissue responses. Many inflammatory and other immune cells are involved in the biological responses. The role of mast cells in mediating host response to biomaterials is still however speculative.

Chapter 1 introduces the background of the study; reviews literature on the previous studies conducted and has sited the main hypothesis and objectives of the present study. Studies on clinically retrieved, failed implants reveal the main reason behind failure as the adverse host response to the implanted material. For any material to survive *in vivo* it should be biocompatible. Literature reviewed reveals the concepts of biomaterials and biocompatibility and the sequences of events of host response following implantation in the body. Even though, primarily most of the materials are considered to be inert, failures following long term implantation have been reported. Most case report involve restenosis of the vascular stents, thrombosis of the vascular implants, aseptic loosening of the joint prosthesis and the excessive fibrosis around the silicone breast implants and shunt materials. Various cell types like macrophages, FBGC, fibroblasts, B cells, T cells etc are found to be involved in the inflammatory response. The role of mast cells as effectors in allergic inflammatory responses has been well elucidated, but their role in inflammatory response to biomaterials including collagen deposition and fibrous capsule formation has yet to be delineated.

The biology of mast cells, their origin, development, occurrence, heterogeneity, mediators and their role in many diseases have been detailed. Recently efforts to find the role of mast cells in modulating host response to implanted polymers, prosthesis, dental implants etc have been initiated. Studies are however still needed to confirm their presence both for the current and recent developments in the field of biomaterials and the biomedical technology. We hypothesize that mast cells

play a significant role in fibrous capsule formation in the peri-prosthetic tissue and their recruitment into the implant site may vary with the type of biomaterial used. This difference may correlate with fibrous capsule thickness around different polymer implants.

Chapter 2 describes the methods and the materials used in conducting the qualitative and quantitative analysis of mast cells around the implant. The samples included in the study are the paraffin embedded tissue sections from different 'gruen zones' of two retrieved knee implants and tissue sections of polymers UHMWPE and Silicone implanted in the gluteus muscles of rats. For qualitative analysis the histological staining methods were used. Hematoxylin and eosin staining was done for identifying cellular morphology, Masson's trichrome staining for identifying fibrous capsule formation and Toluidine blue metachromatic staining for mast cell identification. Immunohistochemical method against the serine protease tryptase was used for confirming the presence of mast cells and its activation. For quantitative studies histomorphometrical analysis using the image proplus software was performed. Statistical analysis using 'Students t test' were done in rat tissue sections for comparing the difference in inflammatory response towards the two polymers.

Chapter 3 details the results of each analysis and briefly discusses the same in the reference to already published scientific literature. Qualitative analysis using different histological staining methods revealed the presence of an inner cellular zone, a middle sparsely cellular collageneous layer and an outer skeletal muscle zone. Numerous perivascularily located round and oval mast cells were observed both at the interface and collageneous layer. Both spindle shaped and degranulating mast cells were also found in both zones. Heavy inflammation with numerous inflammatory cells, presence of particle debri and fibrous capsule formation were also observed. Toluidine blue staining confirmed the presence of mast cells in the inner cellular zone and collageneous layer of periprosthetic tissues. Immunohistochemical analysis of the tissue sections revealed the role of tryptase which is secreted by mast cells in adverse responses. Quantitative analysis using the histomorphometrical methods revealed a denser infiltrate of inflammatory cells around the non articulating surfaces of the

human implant. The average number of mast cells found in the articulating zones and the non articulating zones were almost the same. The fibrous capsule thickness was found to be high near the articulating surface. However no correlation was observed between the number of mast cells or inflammatory cells and the fibrous capsule formation around the biomaterials.

Analysis of rat tissue sections revealed the presence of mast cells and inflammatory cells around both the polymer implants (UHMWPE and Silicone) at all time periods of implantation, but with a higher number of mast cells and the inflammatory cells around the silicone implants as compared to the UHMWPE. The number of mast cells and the inflammatory cells were found to increase slowly with the time being highest at one month post implantation and then decrease with a persistent presence even at three months in case of both polymers..

Chapter 4 summarizes and concludes the present study briefly and gives an outline on future prospects. Our hypothesis about mast cell involvement in the biological responses to the components of knee implants and to the two polymers, UHMWPE and silicone at different time periods proved to be affirmative. However no correlation was established between the number of mast cells and the thickness of the fibrous capsule formation around the implanted biomaterials. For more confirmatory results a wider number of samples may be included and further analysis using different antibodies to the specific antigens and molecular level studies to understand the expression of different genes may be conducted.

CHAPTER 1
INTRODUCTION

1.1. Background

Diseases and dysfunction of organs and organ system are the main challenge that humans continue to face. Even in this century of most modern technologies and facilities, man is not able to completely win over these problems. The use of biomedical devices and implants in the practice of medicine to evaluate, treat, augment or replace any tissue, organ or function of the body is increasing worldwide. A surgical implant may be defined as an object made from a non living material that is inserted into the human body, where it is intended to remain for a significant period of time in order to perform a specific function. Materials used for constructing implants are either natural or synthetic and termed 'biomaterials'.

By definition "A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interaction with components of the living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine". The most commonly used materials for constructing biomedical devices are polymers like polyesters, polyurethanes, silicone, metals like Stainless steel, Titanium, Cobalt-chromium alloy etc and ceramics like Alumina, Zirconia and composites. The most recent development in the field of biomaterial is the use of tissue engineered materials to replace damaged tissues and organs.

The most important factor that distinguishes a biomaterial from any other material is that it should be biocompatible. The sole requirement for biocompatibility in a medical device intended for sustained long term contact with the tissues of the human body is that the material shall do no harm to those tissues, achieved through chemical and biological inertness. Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable, local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial, cellular or tissue response in that specific situation, and optimizing the clinically relevant performances of that therapy. The success of an implant depends on multiple factors and it is necessary to

determine whether failure was inherent to the device or was caused by external factors such as surgical procedure, patient co-operation or rate of healing. Two clinically relevant areas of prosthesis failure are loosening of total joint prosthesis and contracture of breast implants. Materials proved to be biocompatible are used in both. However, long term implantation leads to severe inflammation and fibrosis.

Orthopedic implants, namely total joint replacements for the knee and hip are subjected to mechanical loads and must integrate with the host bone. Failure of these implants to integrate with bone, infection and sepsis and aseptic loosening are serious clinical problems. Aseptic loosening has been found to be due to release of wear debri into the adjacent tissues, severe inflammation followed by the formation of a thick fibrous capsule at the implant bone interface and subsequent osteolysis of adjacent host bone.

Silicone breast implants have been used for breast augmentation and reconstruction since the early 1960s and are either saline-filled or gel-filled implants. Saline implants have a silicone elastomer shell filled with sterile saline liquid. Both types are reported to give rise to local complications which include the formation of a scar capsule around the implant with subsequent contracture, malposition and rupture and/or leakage of the implant. Rupture of the implant may be contained within the fibrous or extracapsular, breaching the capsule leading to generalized systemic complications.

The response of the body to the implant varies widely according to the host, site and species, the degree of trauma imposed during implantation and nature of the implant material. Immediately after an injury, there are changes in vascular flow, caliber and permeability. An acute inflammation occurs and is characterized by the inflow of numerous inflammatory cells and chemical mediators secreted by them. The inflammatory phase gives way to the formation of granulation tissue which culminates in the formation of a fibrous capsule which is comprised of predominantly collagen and few cells like fibroblast and fibrocytes.

The predominant cell type which is recruited during the first several days following injury is the neutrophils, followed by monocyte and then macrophages at later time periods. Then vascular endothelial cells and fibroblast in the implant site proliferate leading to the formation of granulation tissue. The end stage of healing is the formation of a fibrous capsule around the implant. However persistent inflammatory stimuli may lead to chronic inflammation with the presence of mononuclear cells, including lymphocytes and plasma cells and excessive collagen deposition around the implant.

Mast cell is a less studied cell with respect to the biomaterials. They are known to link the innate immune system, which displays a standard set of defenses, with the adaptive immune system, which customizes the body's response to specific attacker. They develop from progenitor cells that in turn arise from uncommitted hematopoetic stem cells in the bone marrow and reside in tissues that interface with the interior environment, and often in proximity to blood vessels and nerves. These cells express the receptor for stem cell factor (SCF receptor or c-kit) that binds to SCF, latter being a major growth factor for mast cells. Today mast cells have assumed importance in multiple biological processes including phagocytosis, processing of antigen, production of cytokines and other vasoactive substances like histamines, protein slicing enzymes such as chymase and tryptase.

Mast cells promote or regulate homeostasis as they contribute to wound healing as well as tissue remodeling. They have been reported to associate with the development of fibrosis. They interact with the fibroblast in a manner that leads to fibroblast activation and subsequent extracellular fibrosis. There is much documented evidence on the role of macrophages in modulating the inflammatory response to biomaterials and their debri. The sequences of events taking place at the inflammatory region of implant site are highly complex and involve different cell types and their secretory products. The role of mast cells in this inflammatory response to biomaterials has still not been elucidated enough. Understanding the importance of mast cells in the fibrous capsule development and collagen deposition adjacent to the implants may help in finding strategies to reduce or prevent such complications.

1.2. Review of Literature

Man and the world, in which he is living is developing rapidly with new and more efficient technologies. The most noticeable development is in the field of medicine. New methods of treating, preventing and diagnosis of diseases with the help of newer technologies combining all fields of science have proved beneficial to human beings. Biomedical engineering is a particular field of medicine that has made tremendous advances in surgery through the use of implantable devices. These medical devices have proved successful in most areas of the body mainly in the cardiovascular and orthopedic systems. A medical device has been defined in the Federal Food, Drug and Cosmetic (FD&C) Act as an instrument, apparatus, implement, machines, contrivance, implants, in vitro reagent, or other similar or related article, intended for use in the diagnosis of diseases or other conditions or in the cure, mitigation, treatment or prevention of diseases or intended to affect the structure or any function of the body and which does not achieve its primary intended purposes through chemical action within or on the body [Williams, 2000].

Materials used in medical devices are selected with the concept that they should be able to withstand all biological responses in the body for a long period of time without any harmful effect on the body. A biomaterial is defined as any systematically, pharmacologically inert substance or combination of substances utilized for the implantation with in a living system to supplement or replace functions of living tissues or organs [Bhat, 2005]. Natural materials, glass, metals, polymers and composites have been used to replace body parts that have been damaged by disease or injury (Table 1). With the introduction of molecular biology, the field of biomedical technology has become interdisciplinary. As per the International Standard for the biological evaluation of medical devices ISO 10993-1 Part I these devices are categorized by their nature and duration of body contact:

1) By nature of body contact

   a) Non contacting devices

   b) Surface contacting devices.

2) By duration of contacts

   a) Limited exposure: Contact up to 24 hours.

   b) Prolonged exposure: Contact extending from 24 hours to 30 days.

   c) Permanent contact: Contact extending more than 30 days.

Table 1: Commonly used biomaterials and their applications [Adapted from Biomaterials: An introduction, Joon Park, 2007]

Materials	Applications
Polymers (Nylon, Silicone, rubber, polyester, PTFE etc)	Sutures, blood vessels, other soft tissues, hip socket, ear nose.
Metals (Ti, and its alloys, Co-Cr alloys, Au, Ag, Stainless steel etc).	Joint replacements, dental root implants, paces and suture wires, bone plates and screws.
Ceramics (Alumina, Zirconia, Calcium Phosphate including hydroxyl apatite, Carbon).	Dental and Orthopedic implants.
Composites	Bone cement, Dental resin.

Biocompatibility is a word widely used in biomaterial science, but there still exist a great deal of uncertainty about what it actually means and about the mechanism that are subsumed within the phenomenon that collectively constitute biocompatibility [Williams, 2008]. Materials used in devices must be safe in addition to effective. It is important to realize that 1) no one material will be appropriate for all medical device applications 2) the material, its composition and degradation product may affect host cells and tissues; and 3) the host environment may also affect material properties and device performances [Bumgardner *et al.*, 2008]. Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a

specific situation [Williams, 1987]. The key to understanding biocompatibility is in the determination of which chemical, biochemical, physiological, physical or other mechanisms becomes operative, under the highly specific conditions associated with contact between biomaterials and tissues of the body, and what are the consequences of these interactions.

The evaluation of biological responses to a medical device is carried out to determine that the medical device performs as intended and presents no significant harm to the patient or user. This evaluation is to predict whether a biomaterial, medical device, or prosthesis presents potential harm to the patient or user by evaluating conditions that simulate clinical use [Anderson, 2001]. The determination of biocompatibility of materials and implant devices involves detailed characterization of the material and extensive testing, first at the cell/tissue level and then in in-vivo animal models and ultimately in human clinical trials. The design and use of biocompatibility testing protocols is provided by a variety of professional and regulatory organizations, including ASTM, ISO, ADA, NIH and FDA. The methods and evaluation criteria for determining biocompatibility are routinely reviewed and amended as additional information is collected.

1.2.1. Biological response to the implanted biomaterial.

All materials intended for application in humans as biomaterials, medical devices, or prosthesis elicit a tissue response when implanted into living tissue. The fate of the material depends on the material characteristics as well as the tissue response. The process of implantation of a biomaterial, prosthesis, or medical device results in injury to tissues or organs [Anderson, 1993]. It is this injury and the subsequent perturbation of homeostatic mechanisms that lead to the cellular cascades of wound healing (Table 2).

Table 2: Sequence of host reactions following implantation of a medical device.

- Injury
- Blood-material interactions
- Provisional matrix formation
- Acute inflammation
- Chronic inflammation
- Granulation tissue
- Foreign body reaction
- Fibrosis/fibrous capsule development

Immediately following implantation, proteins and other biomolecules present in the blood plasma and biological fluids rapidly adsorb onto the surface of biomaterials. In many instances, adsorbed fibrinogen, IgG, and complement fragments mediate leukocyte-biomaterial interactions and subsequent inflammatory reactions [Bridges *et al.*, 2008]. During the acute phase of this foreign body reaction (FBR), circulating polymorphonuclear leukocytes (e.g., neutrophils) are stimulated in response to inflammatory signals released at the implant site. Short-lived neutrophils are then replaced by inflammatory monocytes and macrophages. The layer of surface-adsorbed proteins modulates macrophage phenotype and subsequent functions, including phagocytosis, cytokine expression, and fusion into Foreign Body Giant Cells (FBGCs). Persistent inflammatory stimuli lead to insufficient healing of local tissue at the device interface. The hallmark of a chronic response is fusion of monocyte-derived macrophages to form multinucleated FBGCs, a complex process involving a myriad of molecules [Anderson *et al.*, 2008]. Additionally, fibroblasts recruited to the implant site generate a thick collagenous fibrous capsule around the implant.

The end-stage healing response to biomaterials is generally fibrosis or fibrous encapsulation. All injuries to tissues may give rise to fibrosis and fibrous capsule formation, with very little restitution of the normal tissue or organ structure. Tissues composed of permanent cells (e.g. nerve cells, skeletal muscle cells, and cardiac muscle cells) most commonly undergo an organization of the inflammatory exudates, leading to fibrosis. The fibrous capsule was defined by the presence of collagen bundles, elongated fibroblasts and inflammatory cells arranged in parallel with the implant surface. The foreign body reaction and ensuing fibrous encapsulation result in a physicochemical barrier that severely limits device integration and the *in vivo* performance of numerous devices. The influence of material surface chemical properties on the long-term soft tissue response and fibrous capsule thickness and quality are unclear because of the complexity of the materials, models, and evaluation methods. The choice of material will influence fibrous capsule formation and morphology, but the effects of surface chemistry and extended initial inflammatory response on repair are still unknown [Laing *et al.*, 1967 & Ungersbock *et al.*, 1995].

1.2.2. Long term failure of implants

Materials found biocompatible in initial testing procedures, have been found to fail over long term implantation in the human body. Clinical failures with complications leading to revision surgery and replacement of the device have been reported particularly in case of vascular stents and joint prosthesis.

1.2.2.1 Restenosis of vascular stents

Coronary stents are in wide use (more than 80%) in coronary heart diseases as an alternative for balloon angioplasty and are efficient in preventing complications created during angioplastic surgery. The stent material may be made of nitinol, cobalt-chromium, stainless steel, titanium etc. which are tissue and blood compatible. However, the long-term efficacy of coronary stenting is limited by restenosis, which occurs in 15 to 30% of patients. In-stent restenosis is due primarily to neo intimal hyperplasia. Stent-induced arterial injury and the corresponding foreign body reaction incite acute and chronic inflammation of the vessel wall. The inflammatory response,

in turn, produces cytokines and growth factors that induce multiple signaling pathways to activate smooth muscle cell migration and proliferation and formation of a neointima which causes progressive narrowing of vessel lumen.

1.2.2.2 Thrombosis of vascular implants

Direct exposure to blood in case of mechanical heart valves, vascular grafts, stents and coils used in artherosclerosis, coronary artery diseases and aneurisms respectively are prone to thrombus formation on the material surface. Thrombus formation involves activation of the extrinsic and intrinsic coagulation systems, the complement system, the fibrinolytic system, the kinin-generating system, and platelets. Small diameter vascular grafts used for femoral artery surgery have been unsuccessful due to thrombus formation.

1.2.2.3 Aseptic loosening of joint prosthesis

Total Joint Replacement (TJR) is an excellent surgical intervention in patients with joint diseases like rheumatoid arthritis and as replacement in fractures. Complications include improper surgical technique, infection and aseptic loosening all of which leading to pain and revision surgery. The pathophysiological mechanisms have yet to be defined although increasing evidence indicates that cyclic mechanical loading, production of prosthetic wear particles [Santavirta *et al.*, 1990; Schmalzried *et al.*, 1992] and the ensuing adverse tissue response are important contributors to local osteolysis and bone resorption at the bone-prosthesis interface. An interface membrane develops around the loosened prosthesis with a pseudosynovial layer adjacent to it with numerous macrophages. Macrophages stimulate bone resorption when they phagocytose particle [Murray *et al.*, 1990].The resultant cortical bone loss is considered to be caused by activation and release of a cascade of cell mediators by the pseudosynovial macrophages and other cells capable of phagocytosis of the prosthetic particulate debris.

1.2.2.4 Wear debri

One major cause of medium to long term failure of the implant, mostly orthopedic implants is bone loss or bone lysis (osteolysis) caused by adverse reaction to wear particles generated by artificial joints. Release of wear debri in artificial joints and the ensuing inflammatory response is the major reason for revision surgery. Components of joint prosthesis include the load bearing stem and the articulating part. Titanium and Co-Cr alloy are usually used for the stem due to their inherent bulk properties. Either metal or polyethylene forms the articulating surface. Polymethylmethacrylate is used as a material to secure fixation. Wear particles are continuously generated by articulating motion at the bearing surfaces. Billions of micron and submicron size wear particles are generated every year by the articulating interfaces in the artificial joints. These particles accumulate in the periprosthetic tissues and in interface tissues where adverse cellular reactions, predominantly mediated by macrophages occur, lead to bone resorption and eventually loosening and failure of prosthesis. Although cells of the monocyte or macrophage lineage play the primary role in wear induced osteolysis, many other immigrant and resident cells are also active participants in the bioreactive process. The compelling factors that contribute to osteolysis are related to the number, size, shape, and rate of generation, tissue response and antigenic properties of wear debri particles [Tuan *et a*l., 2000].

The analysis of wear debris in tissues adjacent to total hip prosthesis has substantially enhanced our understanding of the nature of the debris generated from wear and corrosion of the implant materials, and the local reaction to such debris. The mechanism by which wear debri influences the bone is illustrated (Figure 1). The particles surrounding the implant stimulate local cells (fibroblast, macrophages, osteoblasts) to release various chemical mediators associated with osteolysis and implant loosening [Hirokawa *et al.*, 2004]. The production of metallic wear debris has been widely reported in many cases after the arthroplasty. The increase in the use of total arthroplasty in younger patients, the development of new alloys and the use of porous coating must raise concern for the long term effects of the accumulation of wear debris in the body [Langkamer *et al.*, 1992].

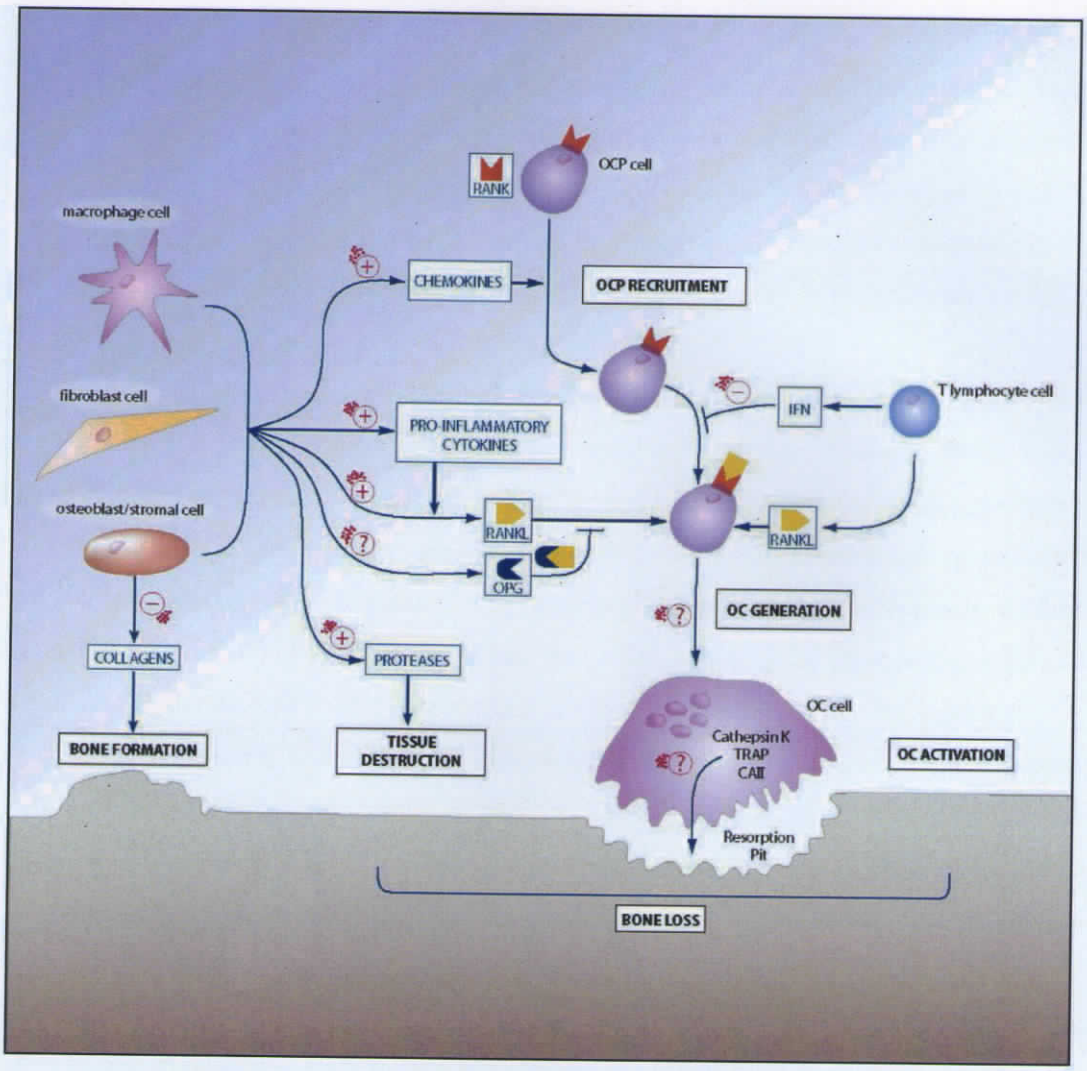


Figure 1: Cellular and molecular regulation of osteoclastogenesis and the influence of wear debris. [Purdue *et al.*, 2006].

1.2.2.5 Excessive fibrosis around breast implants and ventriculo peritoneal shunts made of silicone

Silicone is the generic name for a family of silicon-carbon-based polymers, or chains of molecules. One of the first uses of silicone in a medical implant came in the form of life saving tubes implanted into young children to funnel excess fluid from the brain into the chest cavity, where the fluid could be safely metabolized and excreted. [Ames *et al.*, 1960]. Since these "shunts" were first used, in the late 1950s, silicone in various forms has come to be an important part of many medical devices. It

is used in tracheotomy tubes, in artificial lenses for the eye, in artificial heart valves, and in facial implants for birth defects or reconstructive surgery. It is also found in syringes and intravenous tubing. Today, over two million patients have implanted medical devices made partially or wholly of silicone [Guerrero *et al.*, 1991] most importantly as component of breast implants, either for the reconstruction and augmentation of breast after mastectomy following cancer or for cosmetic reasons.

Though successful initially, severe pain associated with gel bleed/rupture, delayed cancer in implanted women, and autoimmune diseases were reported. Rupture of silicone breast implants is a complication that has been reported with increasing frequency. This may be contained within the fibrous scar or capsule that forms around the implant, or extracapsular, breaching the capsule [Pruitt & Furmanski., 2009]. Implant rupture has been reported in association with closed capsulotomy, which involves manual compression of the breast to break the painful, hard, contracted scar that may form around the breast. Another possible iatrogenic cause of rupture, or potential conversion of intracapsular to extracapsular rupture, is breast compression during mammography [Brown *et al.*, 1997]. Breast implants composed of silicone gel enveloped in a silicone rubber elastomer were introduced in 1963. Local inflammatory processes surrounding Silicone Gel Breast Implant (SGBI) and the migration of silicone fluid from the "gel" to the parenchyma of the breast, lymphatics, and muscle tissues from even intact SGBIs are well established [Marotta *et al.*, 1999]. The migration of silicone fluid may also be problematic in view of uncertainties about silicone oil biocompatibility. In this regard, silicone fluid injections in humans were reported in 1975 to exhibit "adverse systemic effects" including migration with liver dysfunction and foreign body granuloma [Brown *et al.*, 1997].

1. 2.3. Inflammatory cell response to the biomaterials

Irrespective of the site of implantation of a material in the biological tissue there will be inflammatory responses, either acute or if the response persist leads to chronic inflammation. Increased vascular permeability allows the infiltration of numerous inflammatory cells in to the site of implantation. The tissue responses to the

biomaterials mainly show the proliferation of fibroblast accompanied by new endothelial cell forming capillaries and blood vessels. The fibroblastic cells can lay down collagen near the tissue implant interface surrounding the implanted structure by a fibrous capsule and invading its interstices.

Other cells that can invade the tissues are the inflammatory cells like neutrophils, macrophages and multinucleate giant cells. Neutrophils are the first type of cells that immigrate to the site of implanted tissues. The other type of cell is mononuclear phagocyte or monocyte which can ingest many different foreign materials. These macrophages can ingest the metal particles which are the main component of many implants. Multinucleated foreign body giant cells are formed by the fusion of the macrophages that can invade many foreign particles particularly metals, polymers etc. The presence of lymphocytes, plasma cells, eosinophils are sometimes found as a tissue response to the materials. Other major type of cell is the mast cells whose exact function is still a predicament.

1.2.4. Mast cells

Mast cells, involved in both the adaptive and innate immune system play an important role in our body's effective response towards foreign bodies. Mast cells were first observed by Von Recklinghausen in 1863 but were named by the German medical student Paul Ehrlich in 1878 [Foreman, 1993] who called the cells 'mastzellen' (well fed cells) because of the large number of prominent granules in the cytoplasm which he thought represented phagocytosed material. Mast cells are well known for their involvement in allergic and anaphylactic reactions, during which immunoglobulin E (IgE) receptor aggregation leads to exocytosis of the content of secretory granules (1000 nm), commonly known as degranulation, and secretion of multiple mediators. Recent findings implicate mast cells also in inflammatory diseases, such as multiple sclerosis, where mast cells appear to be intact by light microscopy [Theoharides *et al*, 2007].

1.2.4.1 Origin, development, regulation and distribution of mast cells

Kitamura *et al*, 1977 demonstrated in studies in beige mice that mast cells arise from multipotent hematopoietic progenitors in bone marrow. The hematopoietic cell origin of mast cells was confirmed in a number of other studies in which mast cells were grown in culture from bone marrow using factors derived from T lymphocytes. In contrast to other cells of the hematopoietic stem cell lineage, which differentiate in the bone marrow before being released into the circulation, mast cells do not circulate as mature cells mast cells circulate through the vascular system as immature progenitors that then complete their development peripherally within connective or mucosal tissues [Okayama *et al.*, 2006]. Morphologically unidentifiable precursors migrate in the blood and invade connective or mucosal tissues where they proliferate and differentiate in to mature mast cells.

Human mast cell progenitors circulate as mononuclear leukocytes lacking characteristic secretory granules, express CD13, CD33, CD38, CD34, and Kit, but rarely HLA-DR [Castelle *et al.*, 1996].Very little is known about the growth factors and cytokines involved in mast cells development in non mammalian species. Mast cells are the normal residents of connective tissue and are found in highest numbers in areas of the body that interface with the environment, such as the skin, lung and gastrointestinal tract. They occur in virtually all vascularized tissues where they ordinarily reside in close proximity to blood vessels, nerves, smooth muscle cells, epithelial cells, mucus-producing glands and hair follicles [Galli *et a*l., 2008]. Mast cells tend to be located perivascularily and in sentinel locations to respond to noxious stimuli as well as to allergens.

The differentiation and proliferation of mast cells are regulated by two independent mechanisms, both of which were elucidated using rodent models. The first is dependent on a fibroblast derived growth factor (Stem Cells Factor) and the second is dependent on T-cell derived cytokines (Interleukins- 3, 9 and 10) [Michels *et al.*, 1963; Kitamura *et al.*, 1977]. Stem cell factor is an extensively and heterogeneously glycosylated protein, occasionally referred to as the mast cell growth

factor, kit ligand or steel factor. The main sources of SCF in vivo are fibroblast and bone marrow stromal cells [Hill *et al.*, 1998]. The critical importance of SCF/C-kit interactions in the development of mast cell is clearly demonstrated in mutant strains of mice that have chromosomal abnormalities either the W or Sl loci. Further evidence for the role of SCF in mast cell proliferation was obtained from studies in which the soluble form of cytokine was injected into normal animals. Subcutaneous injection of recombinant rat SCF (a biologically active, soluble form of SCF expressed in *Escherichia coli*) into rats resulted in a striking expansion of the mast cell population in the skin, lung, liver, spleen, stomach and small intestine [Tsai *et al.*, 1991]. However in invitro conditions when used alone SCF could not stimulate the development of mast cells from primitive mast cell progenitors, suggesting that its effect was probably dependent on other cofactors and the degree of maturation of the cells [Rennick *et al.*, 1995].

SCF was shown to be a chemotactic agent for mast cells and the interaction between SCF and c-kit could regulate the adhesion of mast cells to the fibroblast or extracellular matrix components such as fibronectin. These functions could be important for controlling the migration of mast cells into appropriate tissues [Kinashi *et al.*, 1994]. SCF also acts as a mast cell survivor factor by preventing apoptosis. It is a regulatory factor for the synthesis of mast cells mediators such as serotonin, heparin and serine proteases [Hill *et al.*, 1998]. Further more recent studies have demonstrated that SCF can also influence the secretory function of the mast cells. In addition to key role in mast cell development, SCF may be directly involved in the regulation of immune responses by modulating mast cell secretory function.

1.2.4.2 Heterogeneity

Although mast cells share many characteristics, it has been known since their discovery that they do not represent a homogeneous population. Mast cells from different tissues exhibit considerable variations in their morphological, biochemical and functional characteristics [Galli *et al.*, 1990]. They exhibit histochemical heterogeneity based on the cytoplasmic granule protein content. Much of the evidence for mast cell heterogeneity arose from studies comparing mast cell

populations in rodent cells lines, spontaneously derived, or resulting from chemical or retroviral immortalization. The presence of more than one type of mast cell in humans is a critical concept relevant to the role of mast cells in health and disease. In rats the mast cells of the intestinal mucosa were distinguished from those in the skin by virtue of their histochemical and secretory properties. This led to the common usage of the terms 'mucosal mast cells' (MMCs) and 'Connective tissue mast cells' (CTMCs) to describe the two sub populations. Two mast cell proteases, chymase and tryptase have been isolated along with a carboxypeptidase and a cathepsin G-like protease. Two types of human mast cell were identified based on the presence or absence of chymase. Cells containing only tryptase (MC_T) were predominant in the lungs, and represented essentially all of the cells in the intestinal mucosa. Mast cells containing both tryptase and chymase, along with the carboxypeptidase and cathepsin G like protease (MC_{TC}), were predominant in the skin and intestinal submucosa. The most important aspect of mast cell heterogeneity, in terms of clinical relevance, is the variation in functional characteristics seen between mast cell subpopulations. This is manifested experimentally as variations in susceptibility to secretagogues or anti allergic drugs [Barret et *al.*, 1993].

1.2.4.3 Mast cell activation

Mast cell activation may be initiated upon interaction of a multivalent antigen (allergen) with its specific IgE antibody attached to the cell membrane via its high-affinity receptor, FcεR1. Cross-linkage of IgE by the interaction of allergen with specific determinants on the Fab portion of the molecule brings the receptors into juxtaposition and initiates mast cell activation and mediator generation and release. Although the process can be triggered by a variety of immunological and non immunological stimuli, the most important signaling pathway in vivo is by aggregation of surface bound immunoglobulin E (IgE) by specific antigen. The IgE receptor known as FcεR1 is a tetrameric complex of non covalently attached subunits, consisting of one α, one β and two γ sub units. Activation of mast cells then result in three types of biological effects, 1) Mast cells undergo regulated secretion in which preformed contents stored in their granules are rapidly released by exocytosis 2) Mast cells enzymatically synthesize lipid mediators derived from precursors stored in cell

membranes and in lipid bodies. 3) Mast cells initiate transcription, translation and secretion of a diverse array of cytokines [Mekori *et al.*, 2001].

The events following aggregation of FcεR1 receptors that culminate in exocytosis are complex and not fully understood, and the mechanism underlying this process are a current focus of mast cell research. Mast cells may also be activated by non immunological stimuli induced by substances such as neuropeptides, basic compounds, complement components, and certain drugs such as opiates. Morphologically degranulation produced by immunologic and non immunologic stimulation appears similar. However, biochemical processes that lead to mediator release may differ. Human mast cells express a multitude of G protein coupled receptors and other recognition sites on their surface which are involved in mast cell activation under physiological and patho physiological condition.

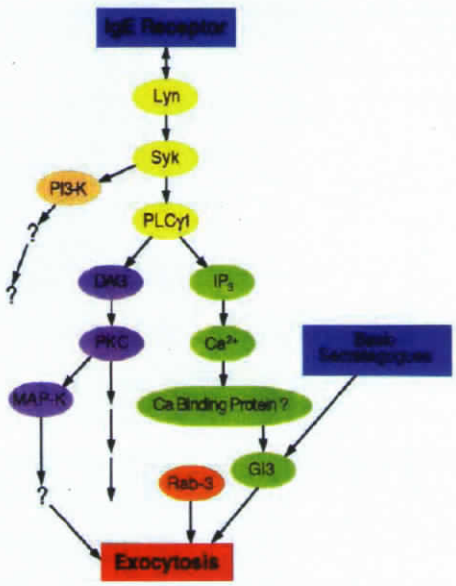


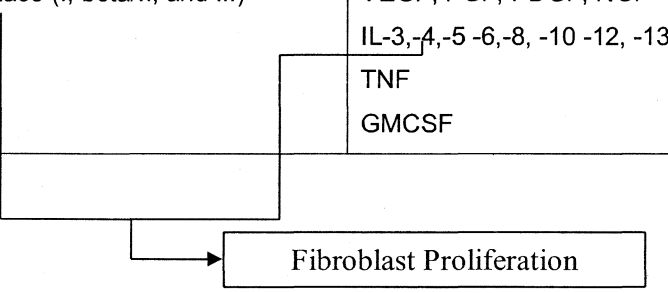
Figure 2: Diagram of signal transduction pathways in mast cells initiated through aggregation of immunoglobulin E (IgE) receptor and resulting exocytosis. PLCyl, phospholipase C-71; PI3-K, phosphatidylinositol5-kinase; DAG, diacylglycerol; IP, inositol 1, 4, &risphosphate; PKC, protein kinase C; MAP-K, mitogen-activated protein kinase. [Ref: Mast cells. Mekori *et al.*, 2007].

1.2.4.4 Mast cell mediators

Upon appropriate activation mast cells release a variety of mediators which are biologically active and have high potency (Table 3). These mediators are both pleiotropic and redundant, that is, each mediator has more than one function, and mediators may overlap in their biological effects. These mediators can be classified as: 1) preformed mediators, that are stored in the cells prominent cytoplasmic granules; including histamine (and in rodents, serotonin), proteoglycans, and proteases such as chymase, tryptase and carboxypeptidase A; 2) *de novo* synthesized lipid mediators, e.g., metabolites of arachidonic acid via either the cyclooxygenase (e.g., PGD2) or lipoxygenase (e.g., LTC4) pathways; and 3) a large number of cytokines, chemokines and growth factors [Marone *et al.*, 1997, Metcalfe *et al.*, 1997, Marshall *et al.*, 2004].

Table 3: Human mast cell mediators [Adapted from Mast cells in the pathogenesis of fibrosis, Gruber *et al.*, 2003].

Preformed easily and eluted	Preformed and granule-associated	Newly generated
Histamine	Heparin and chondroitin sulfate E	Leukotrienes (LTC4, LTD4,
Eosinophil chemotactic	Chymase	LTE4)
factors	Carboxypeptidase	Platelet-activating factor
Neutrophil chemotactic	Cathepsin G	Prostaglandins (PGD2)
factors	Superoxide dismutase, catalase	MCP 1
Superoxide	(rodents)	MIP 1β
Arylsulfatase A	Arylsulfatase B	SCF
Elastase	Procollagenase	TGF
Beta-hexosaminidase	Tryptase (I, beta/II, and III)	VEGF, FGF, PDGF, NGF
Beta-glucosonidase		IL-3,-4,-5 -6,-8, -10 -12, -13
Beta-galactosidase		TNF
Kallikrein-like enzyme		GMCSF



Histamine is the single amine known to be stored in human mast cells, although mast cells of other species are known to store additional amines. For instance, rodent mast cells also store serotonin. Both stabilize mast cell proteases and alter the biological activity of many enzymes. Mast cells in the different species and at different stages of development express varied combinations of granule proteases i.e., the "mast cell granule protease phenotype." Human mast cells contain the same three classes of proteases observed in mouse mast cells. Tryptase is the predominant enzyme and is associated with all human mast cells examined. It is the primary protease of mast cells of the lung, skin, and gastrointestinal tract. It is comprised of a tetramer of 134 kDa, with two subunits of 31- 34 kDa, and each chain possesses a single active site. Five highly homologous tryptases (Icr, 4p) have been cloned and sequenced [Caughey., 1988]. Human mast cell chymase is present in 85% of the mast cells of the skin and intestinal submucosa, but mast cells of the intestinal mucosa and lung have failed to stain with monoclonal antibodies directed against the protease. Carboxypeptidase A is also stored in the mast cell granule complexed with proteoglycans. It is a protein of 35 kDa and acts as a hydrolytic enzyme at neutral pH to cleave the peptide and ester bonds.

1.2.4.5 Mast cells and diseases

Mast cells are most commonly regarded as the key effector cells in the pathogenesis of allergic diseases such as asthma, rhinitis, atopic dermatitis, utricaria, anaphylaxis, and food allergy. The changes in mast cell number in various anatomic sites and/ or evidence of degranulation have been observed in a wide spectrum of innate, adaptive, and pathological immune responses and in a large number of diseases or disease related processes, including delayed hypersensitivity reactions, fibrosis, auto immune pathology, neoplasia inflammation in the rheumatoid synovium and inflammatory bowel diseases. The cytokines which are released after hours after mast cell activation in contrast to the release of mediators indicates that mast cells are not only stores of inflammatory mediators but also important regulatory cells in the immune response. For example, mast cells derived cytokines, especially TNF $-\alpha$, is probably responsible for the leukocyte infiltration seen in the late phase reactions. In addition, the wide variety of biological function attributed to the cytokines secreted by

the mast cells indicate a role in diverse pathophysiological processes such as chronic inflammatory responses, wound healing, angiogenesis, fibrosis, and the acute sunburn response.

Mast cells are uniquely positioned around capillary vessels and may thus play crucial roles in vascular injury and atherosclerosis [Kelley *et al.*, 2000]. First, these cells can directly phagocytose foreign particles (and bacteria) and also express receptors, such as intercellular adhesion molecule ICAM-1 and ICAM-3, CD 43, CD 80, CD 86, and CD 40L, allowing interaction with T and B lymphocytes. Second, they enhance the development of Th2 cells and allow B cells to class switch to IgE. A role as antigen presenting cells has also been proposed for mast cells [Mekori *et al.*, 1999].

Mast cells are increased in numbers in many fibrotic diseases and may play a crucial role in the development of fibrosis [Schaffer L *et al.*, 1995]. The percentages of mast cells in bronchoalveolar lavage fluid from patients with sarcoidosis or interstitial fibrosis are greater than from control individuals, and patients with idiopathic interstitial pulmonary fibrosis show evidence of mast cell degranulation and elevated mast cell numbers [Hunt *et al.*, 1992]. In the kidney tissue of patients with IgA nephropathy, mast cell numbers correlate with the degree of interstitial fibrosis and creatinine clearance. In these kidney tissues, mast cells express tryptase and bFGF [Ehara *et al.*, 1998], which may be partially responsible for the fibrosis observed. The mast cell appears to be the dominant source of bFGF in some patients with pulmonary fibrosis [Inoue, 1996]. The mechanisms behind this relationship between mast cells and fibrosis/ tissue remodeling are unclear. Mast cell products, such as tryptase, TNF-α, and bFGF, induce fibroblast proliferation [Inoue *et al.*, 1996].

However, fibroblasts appear to enhance mast cell survival, suggesting the presence of a bidirectional relationship between these cell types [Church *et al.*, 1997]. Fibroblast expression of SCF and its interactions with c-kit on mast cells may provide one explanation for these observations. Fibroblasts, however, also are closely opposed

to mast cells in fibrotic diseases, suggesting the additional possibility of cognate, cell–cell interaction such as that mediated by CD40–CD40L ligation. To further complicate the picture, mast cells are themselves capable of laying down some forms of collagen and mast cell tryptase can activate collagenases capable of matrix degradation. These data suggest multiple mechanisms by which and multiple levels where mast cells can regulate tissue fibrosis and repair [Williams CM et *al.,* 2000].

A disorder characterized by excessive numbers of mast cells and tissue infiltration by these cells is systemic mastocytosis. Osteoporosis is often a feature of mastocytosis, and mast cells may contribute to bone resorption [Lehmann *et al.,* 1996]. By inducing angiogenesis, the secretion of VEGF and bFGF, and the elaboration of collagenases, mast cells can contribute to tumor pathology and invasiveness [Duncan *et al.*, 1998]. A probable role for mast cells and IgE-mediated pathology has been reported in HIV infection [Marone *et al.,* 2000]. Mast cells may play a role in various arthritides by the release of mast cell mediators (α and β tryptase and histamine) in the joints of various forms of inflammatory arthritis.

Many recent developments in understanding of mast cell biology have led to the finding that mast cells play important role in many cardiovascular diseases. Their presence has been implicated in artherogenesis since they are found in heart and around coronary arteries in human [Bot *et al.,* 2007]. The importance of a possible role of chronic inflammation in cancer has been recently been discussed and the role of mast cells in either promoting cancer formation or inhibiting it is studied. Gouaris *et al.,* 1999 recently reported that mast cells accumulate in adenomatous polyps (in a lympocyte-independent manner) and are required for polyp formation, the initiating step of colon cancer. Although other evidence also suggests that there are some tumor models in which mast cells appear to have roles that favor the host. For example, Sinnamon *et al.*, 2008 reported a protective role for mast cells in colorectal tumorigenesis.

1.2.5. Mast cells in relation to biomaterials

It is well established that biomaterials such as metallic implants can cause sensitivity during exposure to the biological tissues, with the attraction and activation of macrophages. In fact a wide variety of particles can prime macrophages to have a marked increase in their oxidative response [Myrvik *et al.*, 1993]. But recent investigations have found some role of mast cells in the inflammatory response to biomaterials and the subsequent formation of fibrous capsule. But still there is not much evidence to prove their role in the inflammatory response to the biomaterials. Al Saffar *et al.,* 1998, data demonstrated that the accumulation of implant wear particles at the sites of prosthesis joints could enhance the recruitment of mast cells.

One of the studies relating to biomaterials with dental biomaterials demonstrated an inflammatory response at the site of dental implants characterized by the presence of degranulated mast cells [Rezzani *et al.*, 2004]. In a different study done by Christenson et al., 1991 investigating the effect of dms (Dexamethasone) released from Ethylene Vinyl Acetate (EVAc) rods placed in acrylic copolymer capsules and implanted in the peritoneal cavity of rats found there is a correlation between the extent of the tissue reaction and the degranulation of mast cells. A recent study conducted by Klueh U *et al.*, 2010 clearly demonstrates the association and importance of mast cells in implanted Glucose Sensor Function (GSF) in vivo. To confirm the role of mast cells in the inflammatory response to implanted biomaterials further quantification studies are necessary.

Even though the importance of mast cells as mediators in allergic inflammatory responses and various other diseases like scleroderma, pulmonary fibrosis, renal interstitial fibrosis etc have been well elucidated, their role in the biological responses to biomaterials have yet to be investigated. The information about the possible relation between fibrous capsule formation and mast cells may help in developing strategies to prevent or alleviate such complications which often lead to failure of the implant.

1.3. Hypothesis

1) There is a correlation between wear debri generated, mast cell recruitment and fibrous capsule formation in peri – prosthetic tissue.

2) There is a difference in mast cell recruitment in the peri–implant inflammation around different polymers. This difference may correlate with fibrous capsule thickness around different polymer implants.

1.4. Objectives

To carry out a retrospective study of mast cells in two different studies

1) The pseudocapsule around human knee joint prosthesis and the possible relation to excessive collagen deposition.

2) In peri implant tissue around silicone and UHMWPE implants in rat, the former known to elicit collagen deposition and fibrous capsule formation and the latter known for its excellent biocompatibility.

CHAPTER 2

MATERIALS AND METHODS

2.1. Samples:

2.1.1. Clinically retrieved samples

For this study archived paraffin embedded blocks of periprosthetic tissues obtained from two clinically retrieved total knee joint prosthesis were used. The implant in both cases was a cemented Ti implant that had been retrieved after a two year post operative period due to amputation of limbs following a recurrence of osteosarcoma in the patient. The implant was anchored into the tibia and femur with a cemented intramedullary stem. The femoral implant is cemented in the condyle area. Blocks of tissue selected for this study were taken from different 'Gruen zones' (Figure 3) and included tissues adjacent to the articular surfaces of the joint as well as away from the joint.

The components of the prosthesis (as recorded in the laboratory) consists of a femoral and tibial component The femoral component had a metal stem, the proximal end of which was inserted into the femur and the distal end was in the shape of right and left condyles. The condyles were covered with a layer of white smooth polymer and thick hard cream coloured cement material was present on the sides. The tibial component had a metal stem, the distal end of which was inserted into the tibia and the proximal end was expanded with a plateau like surface, lined with polyethylene which articulated with the distal condylar region of the femoral component.

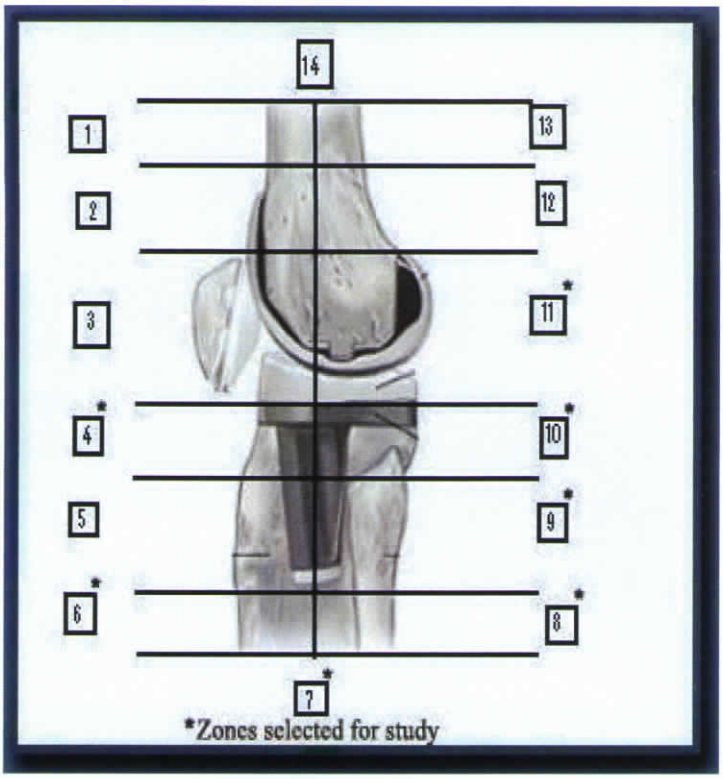


Figure 3: Diagrammatic picture of knee prosthesis showing different gruen zones.

2.1.2. Rat gluteal muscle with implant.

Archived paraffin embedded blocks of muscle tissue from rats implanted with silicone tissue expander and UHMWPE was selected for this study. The blocks were from an earlier study done in the laboratory. Briefly, the study included the implantation of 1.5cm × 1.5cm × 1mm pieces of silicone and UHMWPE in the gluteus muscle of young female wistar rats. The rats were sacrificed at different time periods (3 days, 2 weeks, 1 month, and 3months). Implants were carefully removed and tissue around implant site were fixed in buffered formalin, processed and made into paraffin blocks.

2.2 Sectioning of paraffin embedded tissues

5µm thick sections were cut from the paraffin block (retrieved human implant and rat muscle) on to poly L lysine coated slides (Appendix) for Toluidine blue, Masson's Trichrome, Hematoxylin and Eosin staining and Immunohistochemical analysis.

Materials required:-

Microtome(Leica RM 2225), Floatation bath, Incubator(Memmert), specimen tissues embedded in paraffin blocks, disposable steel knife, micro slides(Gold star), forceps, brushes, wooden stands, ice blocks in ice trays and slide racks.

Method

A disposable steel knife was fixed on to the microtome and its position was adjusted. A floatation water bath was set at 45°C to 50°C. The paraffin block was fixed on to the microtome and cutting surface oriented towards knife. The blocks were trimmed to expose tissue. Sectioning mode was selected in the microtome and a new disposable knife fixed. Ice cubes were applied on the surface of the block and the excess water wiped off gently. Multitude 5 micron thick sections were cut and were carefully removed and floated on to floatation water bath. It was then lifted on to the poly L lysine coated micro slides. The sections were air dried and kept stored in an incubator at 37°C, overnight or at 50°C for 1-2 hours.

2.3. Staining methods

2.3.1. Hematoxylin and Eosin staining

Hematoxylin and eosin staining is usually done to study the architecture of tissue sections at light microscopic level based on the histological affinity of various tissue components. The nucleus stains blue and cytoplasm stains pink or red.

Materials required:

Incubator, Autostainer (Leica Autostainer XL) , Microscope(Nikon., Japan), slide racks, staining troughs, forceps, Isopropyl alcohol of different grades(Merck), Harris hematoxylin solution (Appendix), Eosin (in alcohol or water) (Appendix), Scott's tap water, acid alcohol, Xylene(Merck), Cytoseal ^{TM} 60(Richard Allan scientific), cover slip, identity label.

Method:

Sections were stained with hematoxylin and eosin using the following schedule.

- Xylene I- 10 minutes
- Xylene II – 5minutes
- 90% alcohol – 5 minutes
- 70% alcohol – 5 minutes
- Wash I (with tap water) – 3 minutes
- Hematoxylin solution – 30 minutes
- Wash II (tap water) – 3 minutes.
- Acid alcohol – 10 minutes.
- Wash III (tap water) – 5 minutes
- Scott's tap water – 7 minutes
- Wash IV (tap water) – 5 minutes
- 1% eosin – 5 minutes
- Wash V (tap water) – 15 seconds
- 70% alcohol – 2 minutes

- 100% alcohol – 5 minutes
- 100% alcohol – 5 minutes
- Xylene I – 10 minutes.
- Xylene II – 10 minutes

After these schedule slides were removed from xylene and cleaned with tissue paper without allowing the sections to dry. The slides were then mounted with Cytoseal ™ 60, cover slipped and dried overnight before observing under light microscope.

2.3.2 Masson's Trichrome staining

This method is used for the detection of collagen fibers in tissues in formalin fixed samples. The collagen fibers stain greenish blue and the nuclei stain black in a red background and muscles stain red.

Materials required:

Incubator, microscope, Bouin's fluid (Appendix), Harry's haematoxylin (Appendix), Trichrome stain (Sigma), Cytoseal ™ 60, cover slips, micro slides, distilled water, tap water.

Method:

A working solution of Harry's hematoxylin stain was prepared. The sections were deparaffinised by placing in Xylene and dehydrated through descending grades of alcohol (90% and 70%) for 5 minutes each and then placed in distilled water for about 3 minutes. The slides with sections were placed in the pre heated Bouin's fluid at 56°C for 15 minutes and then cooled in tap water to remove excess yellow colour. It was then stained with Harry's hematoxylin solution for about 5 minutes. After that the slides were washed in running tap water for 5 minutes. Then sections were stained with trichrome solution for 5 minutes and dipped in 0.5% acetic acid. The slides were then rinsed in water, dipped in absolute alcohol, cleared by putting in xylene for about 3 minutes and mounted with the cytoseal. The sections were air dried and viewed under microscope.

2.3.3 Toluidine blue staining

Toluidine blue is a basic dye commonly used for the identification of mast cells. Under the highly acidic conditions only the sulfated proteoglycans remains positively charged and are therefore capable of binding these basic dyes. Binding of toluidine blue to repetitively charged side chains of heparin brings the coloured ionic portion of the dye into close alignment causing a shift in wavelength of the light absorbed. This colour shift is metachromasia and mast cell is seen in bluish purple colour.

Materials required:

Incubator, Toluidine blue stain (Appendix), Isopropanol (Merck), Xylene (Rankem), Cytoseal ^{TM} 60, coplin jar, distilled water.

Method:

The tissue sections were taken on to poly L lysine coated slides and kept in an oven at 50°C for 2 hours, deparaffinised by placing in two solutions of Xylene for ten minute and dehydrated by passing through descending grades of alcohol (90%, 70%) for 5 minutes each and then kept in distilled water for 3 minutes. They were then stained with toluidine blue for 10 minutes at 37°C. The sections were blotted carefully and dipped in absolute alcohol for 10 seconds and then put in xylene for clearing. The slides were mounted with cytoseal and cover slipped and air dried overnight before observing under the microscope. The positive control used for human periprosthetic tissues was nasal gliomal tissue sections. Control used for rat was normal rat muscle tissue sections.

2.3.4 Immunohistochemical analysis

Immunohistochemistry is the method of localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold. Clinically retrieved human samples were stained with mast cell tryptase antibody (Dako) in the dilution 1:100 and downstream visualization done by Supersensitive polymer HRP- IHC detection system.

Materials required:

Incubator, cold chamber, microscope, hot plate or microwave oven, tissue sections, microscopic slides, descending grades of alcohol, Xylene, distilled water, primary antibody (Monoclonal mouse anti human mast cell tryptase, Clone AA1, Dako), Supersensitive polymer HRP IHC detection system (Biogenex, USA), Peroxide block, Protein block, Phosphate buffer saline (PBS) (Appendix), Diamino benzidine (DAB), Hematoxylin stain (Appendix), Scott's tap water (Appendix), mountant (Cytoseal), Citrate buffer (pH 6) (Appendix).

Method:

The tissue sections were taken on to poly L lysine coated micro slides, kept overnight in an incubator at 37°C, deparaffinised by keeping them in two solutions of xylene and dehydrated through descending grades of alcohol and kept in distilled water for about 3 minutes. The positive control used was nasal gliomal tissue sections.

Immunohistochemical staining was then performed using the following protocol.

- The slides with the tissue sections were washed twice with PBS buffer.
- The slides were placed in pre boiled citrate buffer (pH 6) for about 10 minutes for antigen retrieval.
- The sections were then cooled in the buffer itself for about 20 minutes.
- It was then blot dried and marked.

- The peroxide block was then added on to the sections and kept for 10 minutes and then poured off the solution without rinsing.
- The protein blocking solution was added and kept for 10 minutes.
- The solution was poured off without rinsing.
- The slides were then carefully wiped with filter paper.
- Then the primary antibody solution was added on to the sections and incubated at room temperature for 2 hours in a cool chamber (or kept at $4^{o}C$ overnight).
- The slides were then washed with PBS.
- The enzyme (HRP) conjugated anti isotype antibody (secondary antibody, ss label polymer HRP) was added on to the slides.
- It was incubated at room temperature for about 30-45 minutes.
- The slides were then washed twice with PBS solution.
- Then the substrate diaminobenzidine (DAB) was added and kept for 3-5 minutes and washed again with PBS.
- The sections were then counter stained with hematoxylin and kept for 30 seconds to 1 minute.
- Then Scott's tap water was added for bluing and washed off immediately in running tap water.
- The sections were air dried, mounted with cytoseal and cover slipped.
- The results were observed using a light microscope.

2.4. Light microscopy and image analysis

All stained sections were observed by transmitted light microscopy (Nikon Eclipse E 600, Japan). H&E stained sections were analyzed for type of tissue response, presence and type of wear debri and fibrous capsule formation. Sections from peri prosthetic tissue were also examined by polarized microscopy (Nikon Optiphot, Japan) for identification of polyethylene wear debri.

Toluidine blue stained sections were observed by transmitted light microscopy. In the case of clinically retrieved knee prosthesis, images from different zones (selected after qualitative microscopy) at a magnification of 40X of the stained sections were captured with a digital camera (Nikon Digital Camera DXM1200F, Japan). A maximum of 10 images per section were captured. Images were then transferred to a computer equipped with image analysis software image proplus (Media cybernetics, Inc: version 5.1, USA). For rat tissue sections images from five different fields around the implant site were obtained at a magnification of 20X. Histomorphometry was done in two different parameters.

Mast cells were identified in toluidine blue stained section as round oval cells with deep blue granules. Total number of inflammatory cells and the number of mast cells in each high power were quantified. Data was exported to an excel sheet and total number of mast cells per section were calculated as a percentage of total cells. The thickness of the peri implant fibrous capsule in peri prosthetic tissue was measured in Masson's trichrome stained sections using image proplus software. Thickness was measured at a magnification of 1X across the capsule at three different areas and mean calculated.

2.5. Statistical analysis

Statistical analysis using 'Student t test' could not be done in human periprosthetic tissue because less number of sections were used for study. For comparison between the total number of infiltrating mast cells and the inflammatory cells of both silicone and UHMWPE in rat tissue, the 'Students t test' was used. The total number of mast cells and inflammatory cells of all time periods in toluidine blue stained sections of rat tissues were analyzed histomorphometrically and then represented as mean ± SD. A value of $P \leq 0.05$ was used to establish statistical significance.

CHAPTER 3

RESULTS AND DISCUSSION

4.1. Inflammatory and mast cell response in Human periprosthetic tissue.

Tissue sections obtained from the different gruen zones of the retrieved human periprosthesis (Figure 4) and stained with hematoxylin and eosin revealed an inner debri filled dense cellular zone adjacent to the implant, a middle zone of sparsely cellular collagen and an outer zone of skeletal muscle (Figure 5 A&B). The inner zone was densely infiltrated with large round to oval cells identified as macrophages and as small round cells with dense nucleus identified as lymphocytes (Figure 5 C). In occasional sections large foreign body type of giant cells (FBGC) (Figure 5 D) were observed. Numerous spindle shaped cells were found within the outer muscle tissue region and in the inner zone. The infiltration was random with no particular type of arrangement except in few cases, where the inner region appeared undulating with the macrophages arranged in pallisading manner. In some cases, an eosinophilic homogeneous layer was noted all along the inner surface (Figure 5 E). The inner zone was highly vascular with blood vessels ranging from small newly formed capillaries to large vessels (Figure 5 F).

Mast cells are not readily identified by routine histological stains (Bancroft, 2003). Toluidine blue staining revealed numerous mast cells in both zones (Figure 6 A). They were identified by their extensive cytoplasm with deep blue purple granules which distinguished them from other cells (Figure 6 B). They were round, but some were oval and spindle in shape (Figure 6 C). Extensively degranulated mast cells were seen at sites adjacent to the implant (Figure 6 D). Most of the cells were located perivascularily (Figure 6 E). They were seen both in the inflammatory and fibrous capsular region being less in the capsular region. Immunohistochemical analysis confirmed the presence of tryptase positive cells with degranulation in the inflammatory and fibrocapsular region around the prostheses (Figure 7 A).

Numerous foreign body debri were observed in most of the sections, confined mainly to the inner zone. Many of these debri were found in the tissue sections obtained adjacent to the articulating surface of the prosthesis. Morphologically, the debri were of three types. Predominantly black, homogenous debri, both as fine powdery form (Figure 8A) about less than 1μm in diameter and large pieces

(Figure 8B) were present in the dense inflammatory infiltrate in the inner zone in most sections. Powdery debri phagocytosed by macrophages (Figure 8C) as well as FBGC's (Figure 8D) were also observed. The FBGC showed cytolysis (Figure 8E). Occasional large homogeneous grey irregularly shaped cement fragments surrounded by macrophages were observed (Figure 8F). Fragments of refractile material of varying shape and size were also seen in most of the sections (Figure 9 A). These refractile particles, found extremely birefringent by polarized microscopy were identified as polyethelene wear debri in FBGC (Figure 9 B). Areas of clear spaces of more than 1 micron diameter, close to each other were also present and denoted polyethelene debri (Figure 9C). Degree of different wear debri present in peri – prosthetic tissue is represented in Tables 4 & 5.

Masson's trichrome stain showed a thick layer of collagen deposition (Figure 9D) in the middle zone. Sparse collagen was also seen in the inner cellular zone. Skeletal muscle fibres were observed in the outer zone. Collageneous capsule thickness measured by histomorphometry, varied with sections obtained from different zones. Collagen deposition was much more around the articulating zones, 2000 to 4200 μm than adjacent to the non-articulating zones where it was 600 to 1300 μm thick.

Histomorphometrical analysis of inflammatory cells of all types in Toluidine blue stained sections revealed a denser infiltrate around non-articulating surfaces in comparison to that around articulating surfaces (Figure 10). On the other hand the number of mast cells obtained from histomorphometry on toluidine blue stained sections were more around the articulating surfaces (Figure 11). Percentage of mast cells of all inflammatory cells around the articulating surfaces and the non-articulating surfaces were calculated to be almost same. However there was no correlation between the inflammatory cells or mast cell population and collagen thickness in individual sections studied, either from articulating or non-articulating zones (Figure 12 & 13). Qualitative analysis of mast cell presence around different types of wear debri did not reveal any identifiable difference.

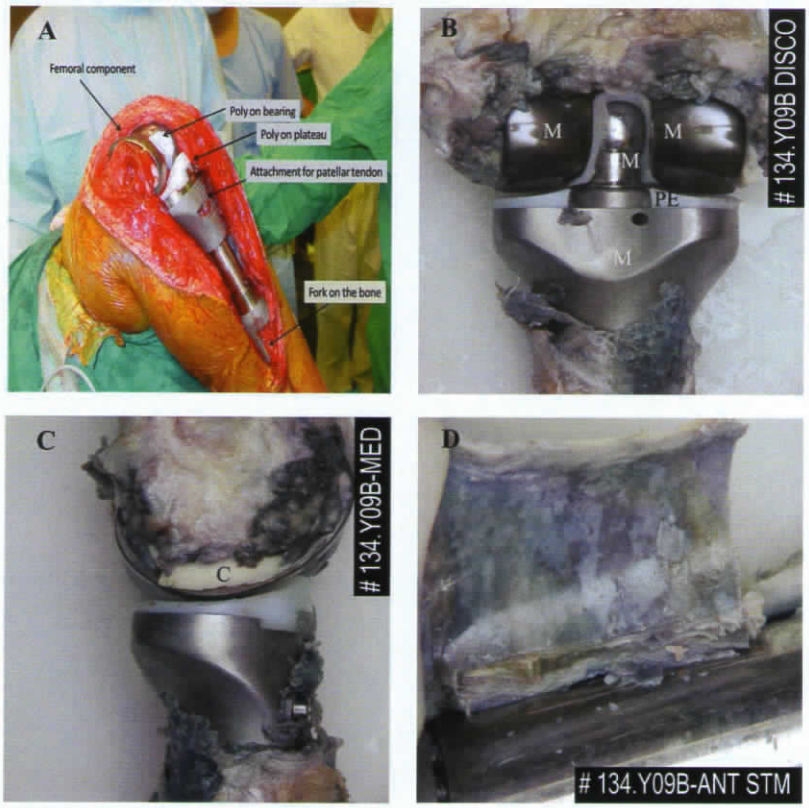


Figure 4: Photographs of Total Knee Joint prosthesis **A.** In situ in patient. **B.** Anterior view of retrieved specimen with metal (M) and polyethylene(PE) components. **C.** Lateral view with cement (C) component. **D.** Internal discoloured surface of peri - prosthetic tissue

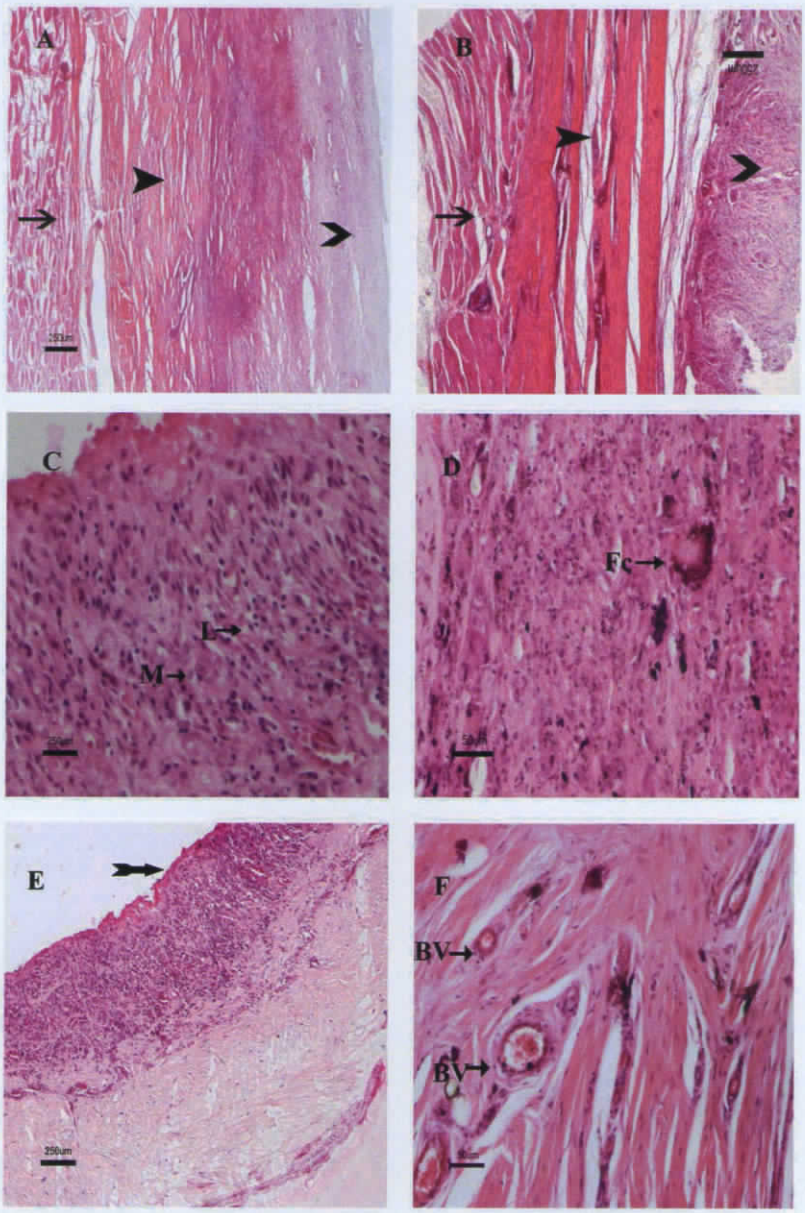


Figure 5: Photomicrographs of peri-prosthetic tissues. **A** and **B**. Inner cellular zone ➤ Middle collagen zone ➤ and outer zone of skeletal muscle →. **C**. Inflammatory infiltrate in inner zone with macrophages (M) and lymphocytes (L). **D**. Foreign body response (Fc) to wear debri. **E**. Eosinophilic homogeneous layer at implant - tissue interface. ➤➤ **F**. Numerous blood vessels (BV) small and large. Hematoxylin and Eosin.

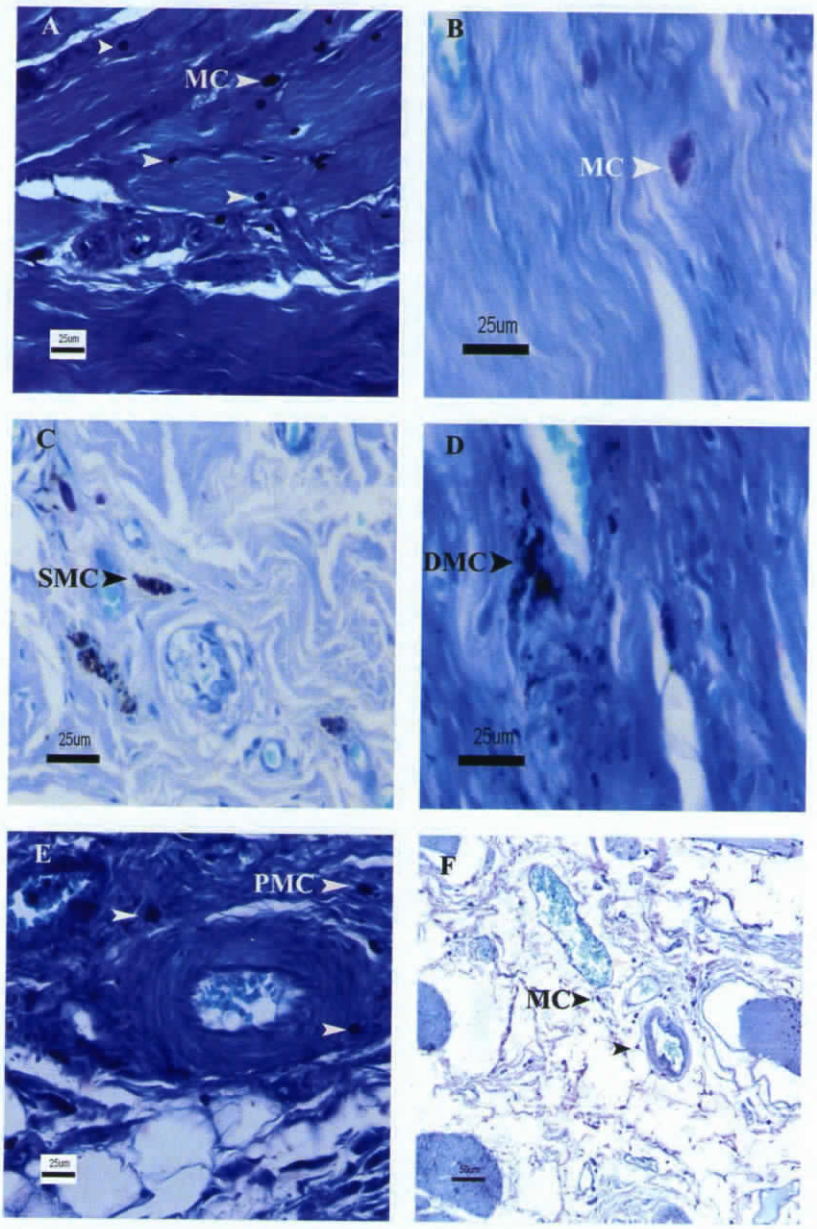


Figure 6: Photomicrographs of mast cells in peri-prosthetic tissue. **A.** Round to oval cells (MC) in middle zone. **B.** Intracellular bluish purple granules (MC). **C.** Spindle shaped mast cells (SMC). **D.** Degranulating mast cells (DMC) with granules in extracellular matrix. **E.** Perivascular mast cells (PMC). **F.** Positive control for mast cells (MC) in human tissue. Toluidine blue

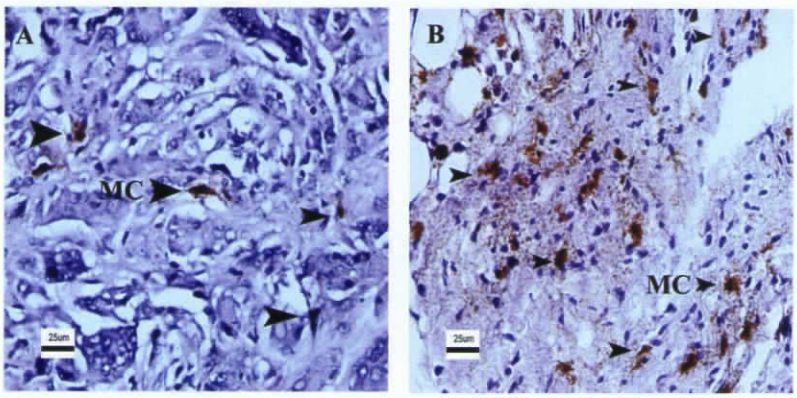


Figure 7 : Immunostaining with monoclonal Mouse Anti - Human Mast Cell Tryptase, Clone AA1 confirmed the presence of Tryptase positive mast cell in **A**. peri prosthetic tissue and **B**. Positive control of human tissue. Hematoxylin counterstain.

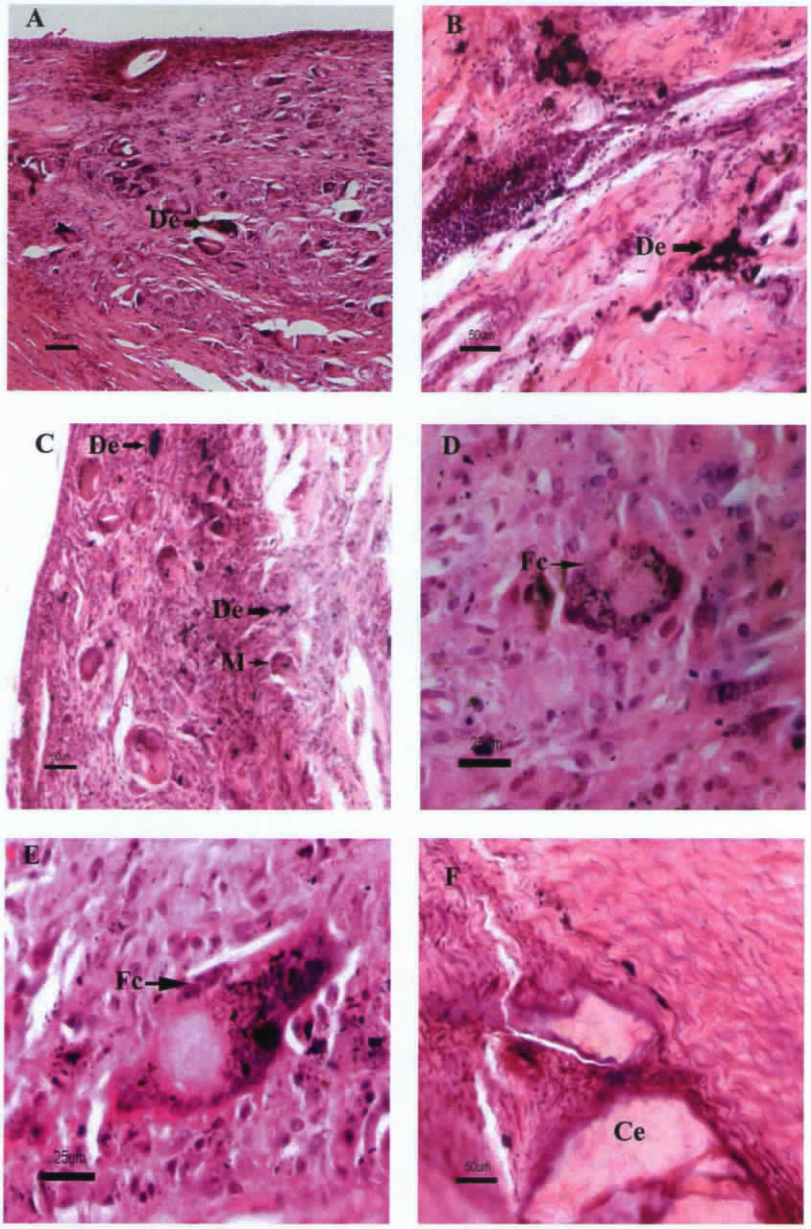


Figure 8 : Wear debri in peri - prosthetic tissue. **A**. Fine metal particles with inflammatory cells (De). **B**. Large black metal particles (De). **C**. Numerous foreign body giant cells (Fc). **D**. Foreign body giant cell with intracellular fine metal particles. **E**. Cytolysis in FBGC (Fc). **F**. Large fragments of cement (Ce). Hemaoxylin and Eosin.

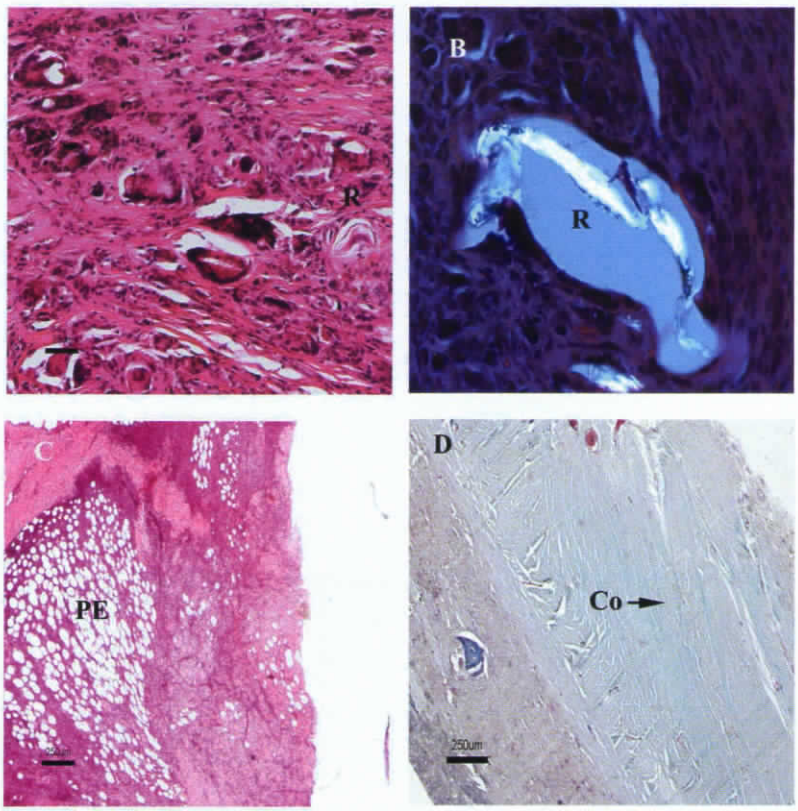


Figure 9: Wear debri in peri-prosthetic tissue. **A**. Refractile poltethylene particles (R) and fine metal debri. **B**. Large birefringent polyethylene particle (PE) in foreign body giant cell. **C**. Clusters of polyethylene particles (R). **D**. Masson's Trichrome stain showing greenish blue collagen (Co) in middle zone. Hematoxylin and Eosin viewed under transmitted light microscopy (A) and polarized light microscopy. Magnification X 200 (B).

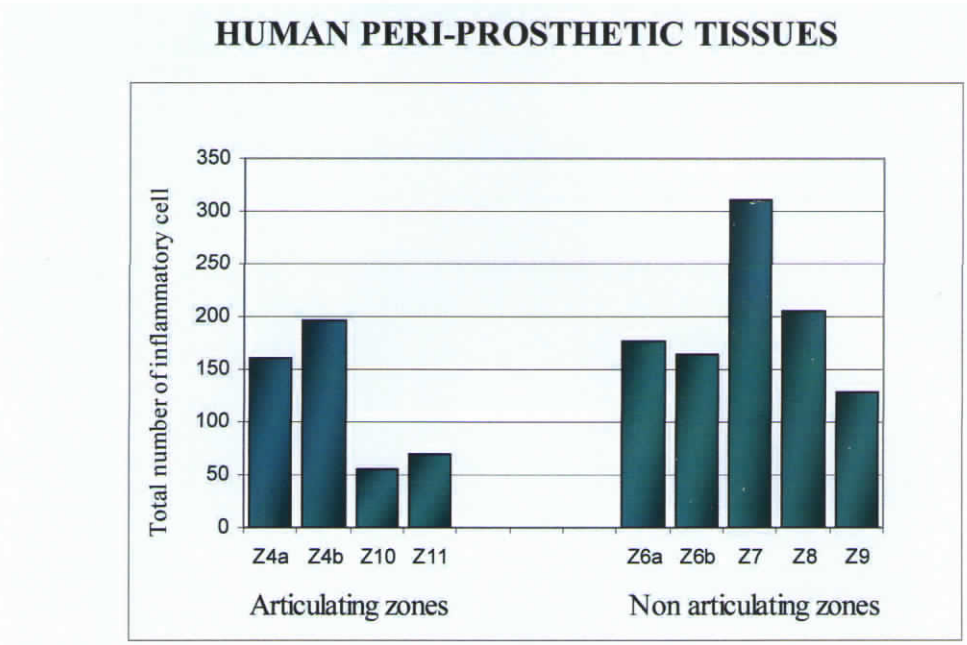


Figure 10: Number of inflammatory cells in peri – prosthetic tissue around articulating and non articulating zones Z in samples a and b.

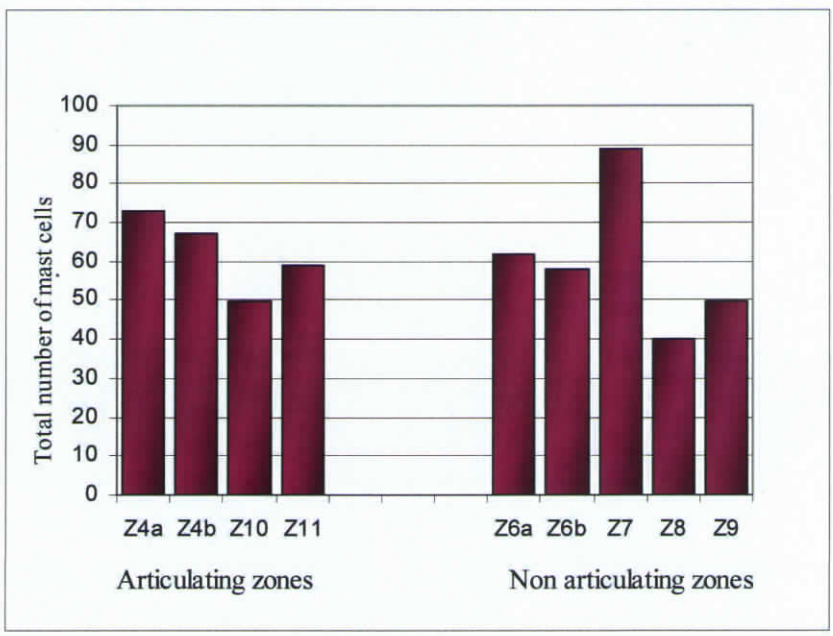


Figure 11: Number of mast cells in peri-proshetic tissue around articulating and non articulating zones Z in samples a and b.

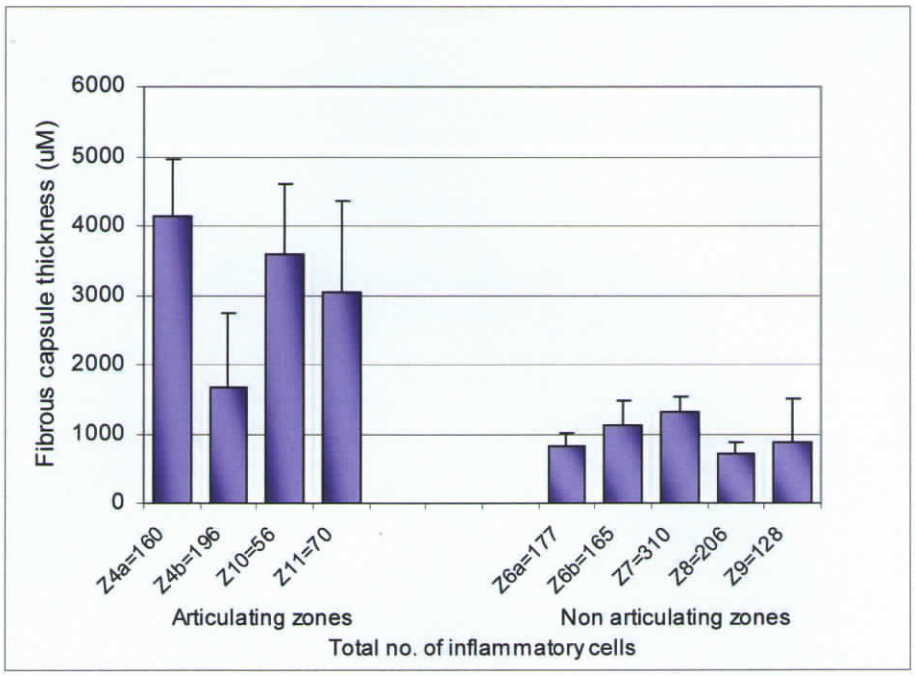


Figure 12: *Inflammatory cell population (X axis) and fibrous capsule thickness (Y axis) in* peri – prosthetic tissue around articulating and non – articulating zones Z in samples a and b.

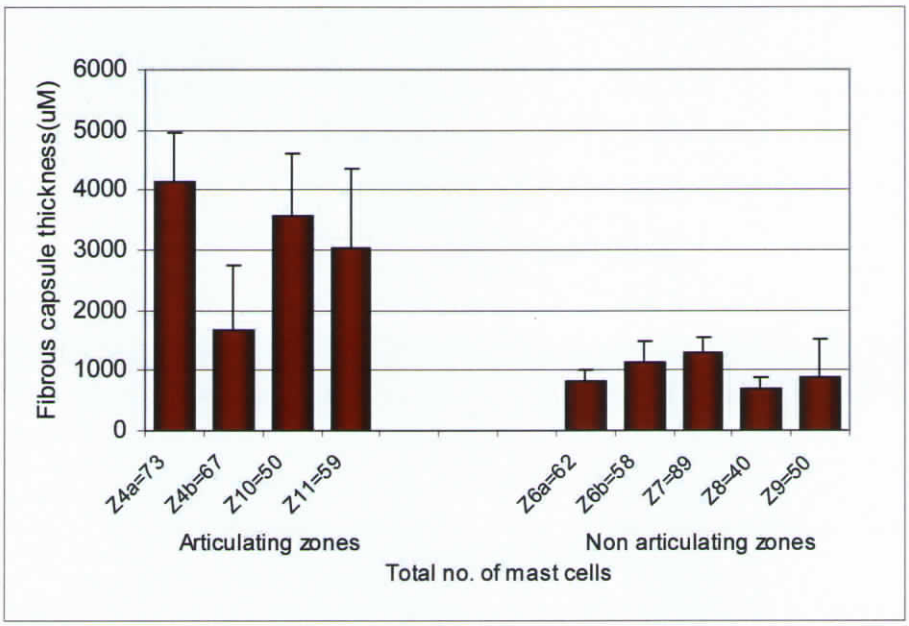


Figure 13: Mast cell population (X axis) and fibrous capsule thickness (Y axis) in periprosthetic tissue around articulating and nonarticulating zones Z in sample a and b.

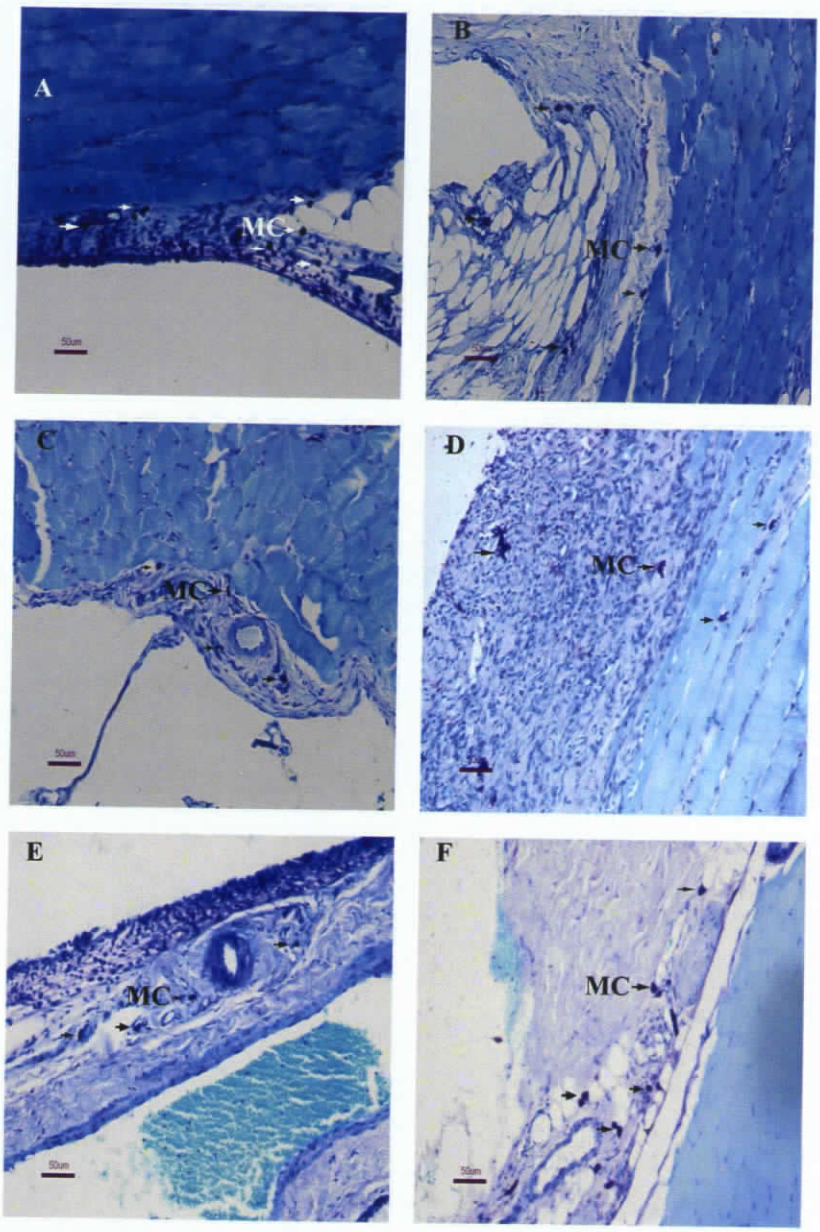


Figure 14: Mast cells (MC) at tissue-implant interface around Silicone (**A,C, E**) and UHMWPE (**B,D,F**) at 3 days (**A,B**), 7 days (**C,D**) and 14 days (**E,F**) post implantation in rat gluteus muscle. Toluidine blue..

Table 4: Degree of wear debri present in peri – prosthetic tissue (Sample A) denoted by mild +; moderate ++; severe +++

Sample A Zones	WEAR DEBRI								
	POLYMER (Polyethylene)			METAL (Titanium)			CEMENT (PMMA)		
3	+			+			+		
3	+				++				
3	+			+			+		
3	+				++				
2	+			+					
11	+				++		+		
12	+				++		+		
11		++			++			++	
12		++			++			++	
6	+				++				
4	+								
4	+			+					
4	+				++				
7	+				++				
7	+			+					
6		++		+			+		
5	+				++			++	
4		++			++			++	
9	+						+		
9	+						+		
8		++							
10	+				++				
10						+++			
9		++			++				

Table 5: Degree of different wear debri present in peri – prosthetic tissue (Sample B) denoted by mild +; moderate '++'; severe '+++'.

Sample B Zones	WEAR DEBRI								
	POLYMER (Polyethylene)			METAL (Titanium)			CEMENT (PMMA)		
7		++				+++			
7	+								
6	+				++				
6		++			++		+		
6				+					
5	+			+					
5				+					
4		++		+					
4	+			+					
10		++			++				
10	+				++				
9		++			++		+		
9	+			+					
8		++				+++	+		
8	+				++				
8		++			++				
7	+				++				
7	+				++		+		
		++			++				

4.2. Inflammatory and mast cell response around UHMWPE and Silicone in rat muscle.

H&E staining of rat tissue sections of different time period revealed an initial inflammation around both Silicone and UHMWPE implants. Acute inflammation composed of neutrophils, macrophages and lymphocytes was present at 3 days post inflammation. The inflammatory region was highly vascular with small and large blood vessels. FBGC were few. Histomorphometrical analysis showed a reduction in total inflammatory cells over 7, 14, 30 and 90 days post inflammation around UHMWPE implants with only few macrophages noted at 90 days (Figure 16). The inflammation persisted around Silicone implants over the entire time period studied with large number of macrophages present at 90 days post implantation. Though there was a higher inflammatory cell response to UHMWPE initially, the response was higher to Silicone at all other time periods post implantation (Figure 16).

Mast cells were identified in Toulidine blue stained sections at all time periods (Figure 14 & 15). These cells were slightly bigger than those found in human tissue and mostly round and oval in shape. Most of the mast cells were located perivascularly either close to or at a distance from implant site. Degranulating cells were also noted.

The average mast cell population as obtained from histomorphometrical analysis of Toluidine blue stained sections was more around Silicone implants than around the UHMWPE implants (Figure 17). Mast cells around UHMWPE were found to increase gradually being highest at 30 days post implantation followed by a decrease and persistence at 90 days (Figure 17). Similar trend was observed around Silicone implants (Figure 17). Statistical analysis of the difference in the mast cell counts as well as a comparison of the values of the UHMWPE and Silicone by Students t test shows a significance of $p < 0.05$.

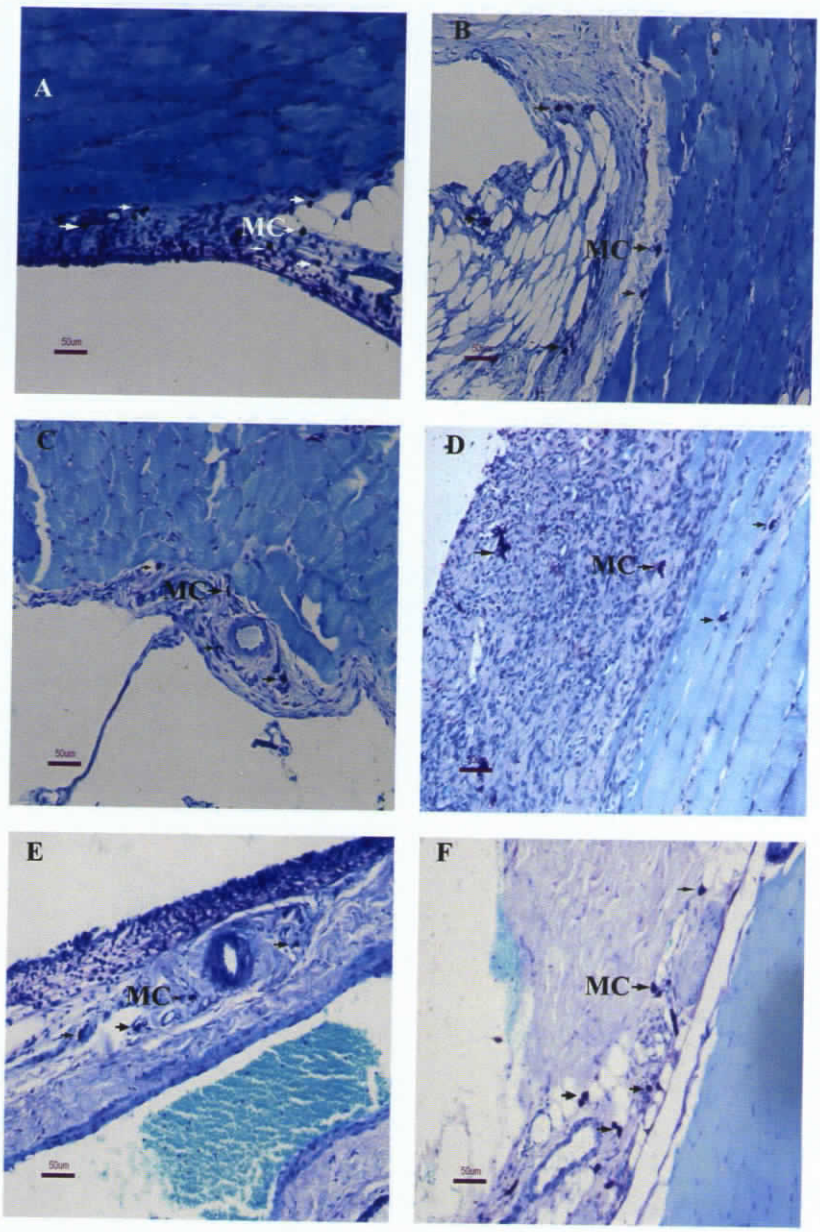


Figure 14: Mast cells (MC) at tissue-implant interface around Silicone (**A,C, E**) and UHMWPE (**B,D,F**) at 3 days (**A,B**), 7 days (**C,D**) and 14 days (**E,F**) post implantation in rat gluteus muscle. Toluidine blue..

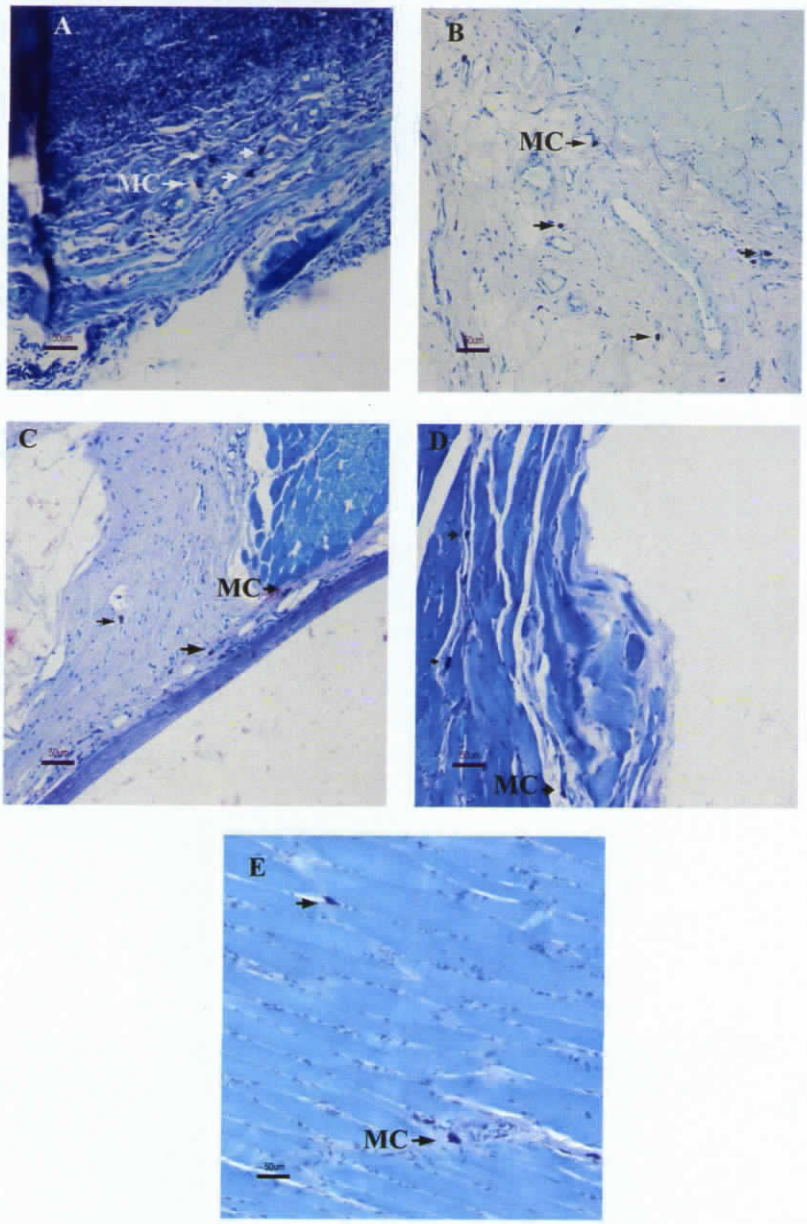


Figure 15: Mast cells (MC) at tissue-implant interface around Silicone (**A,C**) and UHMWPE (**B,D**) at 30 days (**A,B**), 90 days (**C,D**) post implantation in rat gluteus muscle. Toluidine blue.

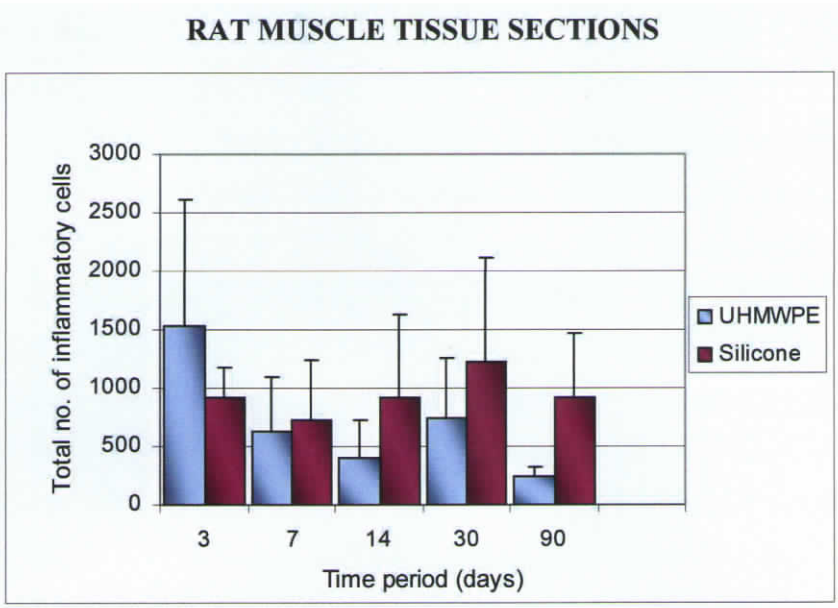


Figure 16: Number of inflammatory cells around the UHMWPE and Silicone implants of different time periods of implant removal. $P < 0.05$ is significant.

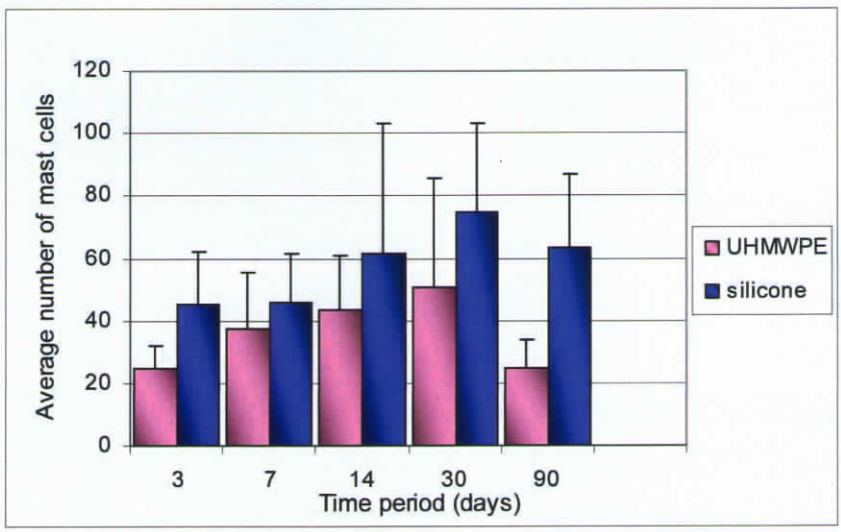


Figure 17: Number of mast cells around the UHMWPE and silicone implants of different time periods of implant removal. $P < 0.05$ is significant.

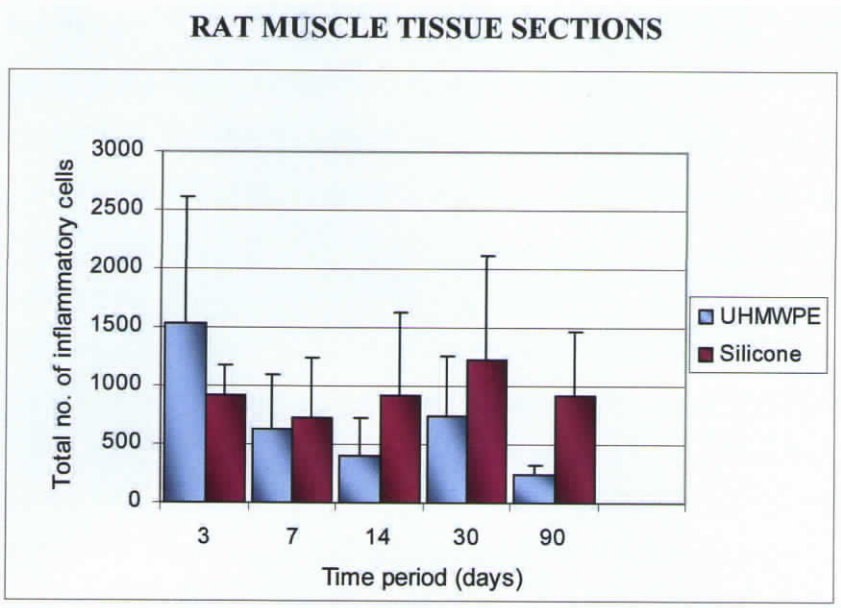


Figure 16: Number of inflammatory cells around the UHMWPE and Silicone implants of different time periods of implant removal. $P < 0.05$ is significant.

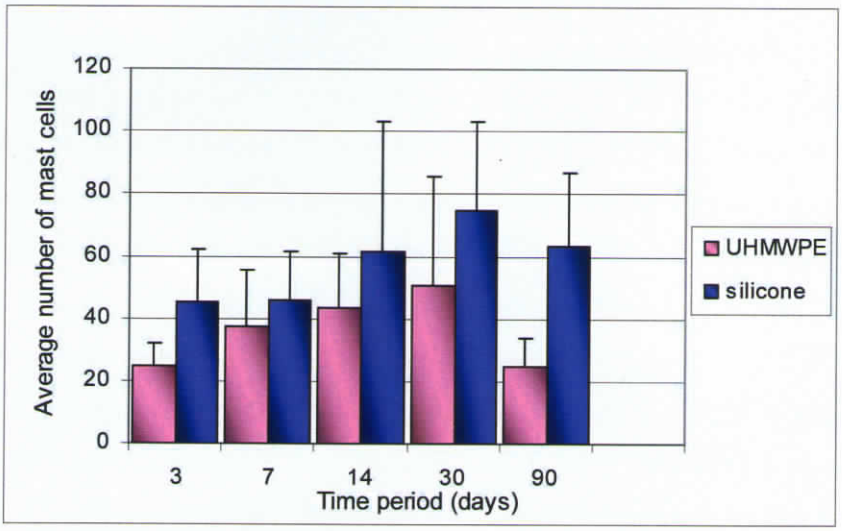


Figure 17: Number of mast cells around the UHMWPE and silicone implants of different time periods of implant removal. $P < 0.05$ is significant.

3.2. Discussions

3.2.1. Human periprosthetic tissues

The present retrospective study examines in detail the biological response around two retrieved custom made total knee prosthesis and has focused on the recruitment of mast cells in the interfacial membrane. Custom made prosthetic replacement, is a common method of limb construction after surgery for bone tumors. It has become apparent that aseptic loosening was replacing infection as the principal mode of failure in such cases [Ward *et al.*, 1991] and the most important factor which has been found to contribute to loosening has been the adverse tissue response to particular wear debri [Toumbis *et al.*, 1997]. The inciting material usually consists of wear particles derived from various components of the implant including polyethylene, bone cement in the form of PMMA, or metal [Jones & Hungerford, 1987]. The study was confined to specific gruen zones [Gruen *et al.*, 1979] to enable denoting of tissue areas related to articulating and non- articulating surfaces.

The investigation demonstrates the definite presence of mast cells in the peri-prosthetic tissue around the prosthesis. Most histological studies of the interfacial membrane adjacent to implants till date have been confined to light microscopy with cell identification being made on morphological characteristics in Hematoxylin and Eosin stained sections. It is well known that mast cells are not readily identified by routine histological stains. Toluidine blue identified numerous mast cells in the inner cellular and middle dense collagen layer in the interfacial membrane in most sections. This was confirmed by immunohistochemical staining for mast cell tryptase. The most abundant protein stored in mast cell secretory granules is endopeptidases, which are released during exocytosis. Hence, immunohistochemistry and immunoassay procedures using antibodies directed against these enzymes are useful experimentally in assessing mast cell locations, numbers and activation [Caughey, 2007]. Numerous degranulating cells were also observed.

Wear debris are continuously being generated by the articulating motion of the prosthetic surfaces. Wear is defined as the removal and relocation of material arising from the contact of two solids. There are four distinct type of wear, namely adhesive, abrasive, corrosive and surface fatigue wear. Adhesive wear occurs when two solids slides over each other such that the fragments are pulled off one surface and adhere to other. Abrasive wear occurs when a rough, hard surface slides over a much softer surface and forms grooves. Corrosive wear involves the wear of the surface oxide films which would otherwise inhibit corrosion, thereby accelerating corrosive attack and surface fatigue wear occurs under repeated loading and sliding cycles [Williams, 2000]. Tissue response to this wear particles differ depending upon the material. But as a common mechanism most of them causes inflammatory responses recruiting macrophages, neutrophils, lymphocytes, foreign body giant cells and fibrous capsule formation.

Extensive studies on the pathogenesis of aseptic loosening of joint prosthesis have revealed heavy inflammatory response to wear debri generated by the implant components. This inflammation is composed predominantly of macrophages and multinucleated giant cells, fibroblasts, vascular tissue and lymphocytes [Pizzoferato *et al.*, 1991 & Dicarlo & Bullough, 1992]. Studies have revealed that macrophage is the predominant cell type with respect to biomaterial particles in inciting periprosthetic inflammatory bone loss [Tuan RS, 2008]. In vitro studies using wear particles from retrieved tissue and molecular studies on cell-cell interactions predominantly cytokines released from macrophages and lymphocytes and their relation to host bone osteolysis have revealed activation of osteoclasts leading to osteolysis. Large particle size foreign bodies (>10uM) induces the fusion of macrophages to form FBGC's. These FBGC's acts as frustrated phagocytes and can release mediators of degradation such as reactive oxygen intermediates (ROI's), degradative enzymes and acid in to the biomaterial surface such that immediate buffering or inhibition of these mediators is delayed or reduced [Anderson, 2008].

Dense collagen deposition is an integral component of the interfacial membrane, which leads to loosening. However, even though strategies are being worked out to identify different molecules for prevention of wear particle release and prevention of the cellular response, not much has been investigated into the fibrosis and its relation to the cellular response, particularly mast cells. Fibrous capsule is formed as an attempt by the body's defense mechanisms to wall off the implant material from other region. The role of mast cells in the pathogenesis of fibrosis in disease state has been extensively reviewed [Gruber, 2003]. Mast cells are generally considered as inflammatory. However, recent review suggests it to have both pro and anti-inflammatory roles [Caughey, 2007]. Literatures on mast cells activation by wear particles are few [Al Saffar *et al.*, 1998; Hansen *et al.*, 2005].

The serine protease tryptase is suggested to be involved in tissue remodeling. It has also been shown to stimulate type I collagen synthesis [Cairns & Walls, 1997] as well as activate precursors of the matrix metalloproteinase collagenase and stromelysin [Gruber *et al.*, 1989; Lees *et al.*, 1994]. It is almost exclusively produced by mast cells [Hauser *et al.*, 1999]. Tryptases are known to exert a mitogenic and fibroproliferative effect and act mostly via activation of protease activated receptor 2 (PAR 2), which are normally expressed in response to fibroblast growth factor. Frungieri, 2002 reports that activation of PAR 2 by tryptase leads to increased expression of Cyclooxygenase 2 (COX2), a key enzyme in the biosynthesis of prostaglandins and consequently to enhanced prostaglandin synthesis. Importantly it seems that prostaglandins, including PGDF2 can regulate fibroblast proliferation and type I collagen production.

In the present study a qualitative analysis showed numerous tryptase positive cells both in the inner cellular zone as well as in the dense collagen layer. However, quantitative analysis of the mast cells and the thickness of the collagen did not show any positive correlation. This may be explained by the contradictory functions of the peptidases in activation of collagen synthesis as well as of collagenase. Furthermore, mast cells derived IL-4 can stimulate fibroblast through the amplification of the action of obligatory fibroblast growth factors such as FGF or PDGF [Trautmann *et al.*,

1998]. Hence, further in depth study is required at the molecular level to investigate the role of mast cells in collagen deposition in peri-prosthetic tissue around implants in aseptic loosening.

3.2.2. Rat muscle tissue sections

The histomorphometric study of mast cell recruitment in response to different biomaterials provides evidence that the recruitment varies with material characteristics. Silicone induced a much higher number of mast cells than UHMWPE. The implantation of a material in the body elicits an inflammatory host response, with cell-cell interaction through activation and release of cytokines [Anderson, 2001]. The composition of inflammatory cells varies with time with neutrophils, macrophages, lymphocytes and fibroblast being predominant in the initial acute phase. At later time periods neutrophils are absent, there is a reduction in number of macrophages and lymphocytes and final repair takes place with the formation of a fibrous/collageneous capsule around the implant. This cellular response depends to a large extent on materials characteristics [Williams, 2000].

In general the biocompatibility of a material with tissue has been described in terms of the acute and chronic inflammatory responses. Acute inflammation persists only for a short period of time lasting from days to months and their main characteristics are exudation of fluid and plasma proteins and the emigration of leukocytes. If inflammation persists it will lead to chronic inflammation which has been used to describe the foreign body reaction where inflammatory cells like monocytes, macrophages and FBGC's are present at the implant interface. This type of inflammation is usually less homogeneous and confined only to the implant site. Numerous blood vessels were found to be formed within the tissues adjacent to the implant indicating angiogenesis and studies reveal that degranulating mast cells are capable of stimulating angiogenesis

Devices with components found biocompatible by standard testing procedures have been found to fail for various reasons following long term residence in the human body. Breast implants made of silicone have been found to elicit extensive collagen deposition and formation of a thick fibrous capsule leading to pain and failure of the implant [Silver *et al.*,1999]. Literature review on studies carried out on biologic evaluation of this capsular tissue has been confined to immune cells with varying results [Williams, 2000]. Investigation into the role of mast cells in the inflammatory response to biomaterials has been occasional. Tang *et al.*, 1998, found evidence in *in vivo* experiments that biomaterial mediated acute inflammatory response are both histamine and mast cell dependent. Histamine is found to be a potent stimulator of fibroblast proliferation and collagen synthesis. Mast cells degranulation and release of histamine has been shown to play an integral role in recruiting phagocytes, including macrophages into the site of implanted biomaterial.

Our study found mast cell to be present at all time periods around both silicone as well as UHMWPE. But this study has revealed a difference between the numbers of cells recruited at the implant interface to both materials which are polymers. An earlier study in this laboratory has found a difference in the fibrous capsule thickness around both the materials with the collagen deposition being more around silicone than UHMWPE [Joseph *et al.*, 2010]. Degranulation of mast cells has been earlier correlated with fibroblast proliferation and collagen deposition in the rat lung [Watanabe *et al.*, 1974, Vergara *et al.*, 1987]. Hence, it is important to carry out further investigation into the interrelationship between mast cells and collagen deposition around silicone. These investigations would help in development of strategies to prevent clinical complications in the use of an otherwise useful biomaterial.

CHAPTER 4

SUMMARY &CONCLUSION

4.1. **Summary**

Adverse biological response of the body to implanted biomaterials remains a reason for failure of medical implants. The present work was undertaken with the aim of understanding the role of mast cells in fibrous capsule formation and collagen deposition around the biomaterial and the difference in mast cell response to different materials.

Data and results obtained from our study reveals that mast cells have an important modulatory effect in the inflammatory responses and suggest their probable role in the deposition of collagen in the interfacial membrane. The response of mast cells at the articulating and non articulating surface of the retrieved implant was same as the percentage of these cells to the inflammatory cells were almost same at the two sites. At the same time the number of inflammatory cells was observed more around non articulating zones. But no correlation was observed between the fibrous capsule formation and the infiltrating mast cells or inflammatory cell number. Fibrous capsule was observed to be thicker around the articulating than the non articulating zones.

The data obtained from the histomorphometrical analysis of rat tissue sections entails that mast cells are involved in the inflammatory response to both the polymers (UHMWPE and silicone) in all time periods of implantation. The number of both mast cells and inflammatory cells increased with the time period being highest at 30 days and then decreased. These cells were found to be more in number around the silicone implants as compared to UHMWPE. Statistical analysis using 'Student t test' also showed significance for this result.

4.2. Conclusion

In conclusion thick fibrous capsule was seen in both studies ie. in human and rat studies and mast cells were also seen in large numbers. Mast cells were found to have a modulatory effect in the biological responses to the implanted biomaterials particularly in inflammatory responses and in collagen deposition. However no correlation was established between the fibrous capsule formation and the mast cells or inflammatory cell number. The presence of tryptase positive mast cell suggests a modulatory effect in the biological responses to the implanted biomaterials particularly in inflammatory responses and in collagen deposition. It was observed that there is a difference in the mast cell recruitment depending upon the type of implant material.

4.3. Future prospects

To better understand the pathomechanism of wear debri induced inflammation larger sample number involving different biomaterials may be used. Immunohistochemical studies using different antibodies targeting different antigens may be used to further the understanding of mast cell mediators and their role in stimulating different cells and cytokines. Molecular level studies would help to analyze the expression levels of different genes during mast cell activation which would add to the current knowledge about the mast cells and their crosstalk with different immune cells.

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APPENDIX

All the chemicals used were of laboratory grade

Phosphate Buffer Saline (PBS)

NaCl	8g
Na_2HPO_4	1.15g
KCl	0.2g
KH_2PO_4	0.2g
Distilled water	1000ml
pH	7.4 ± 0.2

Dissolved the salts in 700ml of distilled water, adjusted the pH with 0.1N Hcl/NaOH and made up to 1000ml.

Harris Hematoxylin Stain

Hematoxylin powder	5g
Absolute alcohol	25ml
Ammonia/ Potassium alum	50g
Mercuric oxide	1.25g
Glacial acetic acid	20ml

The hematoxylin powder was dissolved in water by gentle heating. The two solutions were mixed by pouring the hematoxylin solution into the alum solution while hot. Boiled rapidly while continuously stirring the solution. To this added mercuric oxide slowly. The vessel was plunged into a basin of cold water. 20ml of glacial acetic acid was added to it and the solution filtered and stored.

Eosin stain

Water soluble eosin	0.1%
Eosin	10g
Acetic acid	0.5ml
Distilled water	1000ml

Eosin was dissolved in 700ml of distilled water. The solution was made up to 1 litre with distilled water. To this added 0.5ml of acetic acid and a pinch of thymol. It can then be filtered and stored.

Acid Alcohol

Isopropyl alcohol	700ml
Conc. HCl	10ml
Distilled water	300ml

700ml of isopropyl alcohol was made up to 1 litre with distilled water. Pipetted out 10ml of this solution and discarded. To the remaining alcohol solution 10ml of concentrated HCl was slowly added.

Scott's tap water

K_2HCO_3	2g
$MgSO_4$	20g
Distilled water	1000ml

K_2HCO_3 was dissolved in a beaker with a little water. Dissolved MgSO4 with distilled in a separate beaker. Poured the dissolved bicarbonate into the MgSO4 solution and mixed well. The solution was made up to 1 litre with distilled water.

10% Neutral Buffered Formalin

Na_2HPO_4	0.5g
$NaHPO_4$. H2O	4g
Formalin	100ml
Distilled water	900ml

To 900ml of distilled water dissolved Na_2HPO_4 (anhydrous), $NaHPO_4.H_2O$ and 100ml of Formalin. The pH of the solution was adjusted to 7. The solution was made up to 1litre with distilled water.

Toluidine Blue stain

Toluidine blue	1g
Isopropanol	50ml
Distilled water	100ml

1g of toluidine blue was dissolved in 50ml of isopropanol and then made up to 100ml with distilled water.

Bouin's Fluid

Saturated aqueous picric acid	70%
Formaldehyde	25%
Acetic acid	5%

To 70% saturated aqueous picric acid, added 25% formaldehyde and 5% acetic acid and mixed well.

Citrate buffer

Citric acid (Anhydrous)	1.92g
Distilled water	1000ml

1.92g of Citric acid (anhydrous) was dissolved in 700ml of distilled water. Adjusted the pH to 6.0 with 1N NaOH and made up the solution to 1 litre with distilled water.

Poly-L-Lysine coated slides

Poly-L-Lysine solution (0.1% w/v in water) (Sigma-Aldrich, USA) was diluted 1:10 with deionised water. This was then coated on clean microslides by smearing the solution on the slide. The slides were then kept for drying at 56°C in an incubator for one hour.