The Effect of Bone Marrow Stem Cells on The Healing of Colonic Anastomosis in Rats

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General Surgery

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CERTIFICATE

This is to certify that the thesis entitled "The Effect of Bone Marrow Stem Cells in The Healing of Colonic Anastomosis in Rats" is based on the work carried out by Dr. Veracious Cornerstone Wann in partial fulfillment of the requirements for MS (Branch I) General Surgery examination of the Tamil nadu Dr. MGR Medical University to be held in March 2008.

The candidate has independently reviewed the literature and carried out the techniques towards completion of the thesis. This thesis has not been submitted for the award of any degree or diploma of any other university.

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CONTENTS

1.0 INTRODUCTION:

The concept of cell regeneration dates well back to ancient history, when Prometheus transgressed the Laws of the ancient gods and stole fire for mankind. He was chained to Mount Caucasus, where a vulture preyed on his liver daily which renewed (regenerate) as quickly as it was devoured[1].

Wound healing is a never ending process in the human body. Cells in the skin, lungs, intestinal mucosa regenerate everyday from self renewing progenitors cell located at specific locations in that organ (eg, intestinal mucosal crypts). These nests of progenitor cells are called *niches* (*Fr*. recess). The progenitor cells in tissue organs are mesenchymal stem cells[2].

The concept of organ specific regeneration where cells give rise to their own 'type' has been challenged. These mesenchymal stem cells can be multipotent, resembling pluripotent cell population derived from embryos. Plasticity, the ability of a stem cell of a particular cell line to differentiate into a cell of different germinal layer (eg, bone marrow, a mesoderm into nerve tissue, an ectoderm and vice versa) has enabled researchers to explore this potential of tissue repair in spinal cord injury and acute myocardial infarction.

Colonic anastamosis is a commonly performed operation in general surgery. Despite improvements in suture materials, techniques,

mechanical aid and various nutritional factors, intestinal anastamosis continues to be complicated by dehiscence and stricture formation. Anastamotic dehiscence following colorectal operation is a significant cause of morbidity and mortality in the perioperative period. Low colonic anastomoses have clinically significant leakage rates of around 16% with radiologically demonstrable ones ranging from 27 – 49% Various factors involved in the healing process are not completely understood[3]. This study proposes to look at the role of bone marrow cells in the healing of colonic anastamosis in rats.

Mesenchymal stem cells derived from bone marrow has been used extensively in bone marrow transplants for various indications. Cultured mesenchymal cells ensure the role of stem cells in the healing process but bone marrow cells can be directly innoculated into injured tissue without culture as marrow tissue has a high concentration of multipotent hemopoietic cells.

2.0 AIMS AND OBJECTIVES:

To assess whether Bone marrow derived stem cells when injected into colonic anastomosis-

- Provide adequate cells and response for repair.
- Improve the mechanical strength of colonic anastomosis.

3.0 REVIEW OF LITERATURE

3.1 Introduction-

Stem cell research is one of the most fascinating areas of biology today. However most of the research and applications are restricted to the laboratories and animal models. The application of cell based therapies to treat disease is referred to as regenerative or reparative medicine[4].

3.2 What are stem cells?

Stem cells are unspecialized cells that are capable of prolonged self renewal. Under certain physiological and experimental conditions they can be induced to multiply into specialized cells and at the same time preserve some of the their progeny for future renewal. Specific factors and conditions are required as well for these cells to remain unspecialized and this allows them to be grown in the laboratories.

There are two stem cells sources that have therapeutic potential-

1. Embryonic stem cell
2. Adult stem cell

3.2.1 Embryonic stem cells

Embryonic stem cells as their name suggests, are derived from four or five days old blastocyst stage of embryos. These stem cells can be cultured and passed on for further use and research. These embryonic cell lines are pluripotent and can be preserved to be used commercially.

Undifferentiated embryonic stem cells express Oct-4 protein on their surface which is a laboratory test to identify undifferentiated cells.

3.2.2 Mesenchymal stem cells

An adult stem cell is an undifferentiated cell found among differentiated cells located in what is called *niche*[5]. The primary role of adult stem cells are to maintain and repair tissues in which they are found eg, bone marrow, intestinal crypts, basal layer of epidermis, etc. They are also called somatic stem cells. Bone marrow stem cells has been in therapeutic uses for the last 40 years in the treatment of various hematological conditions. Stem cells located outside the bone marrow are called tissue stem cells.

3.3 Bone Marrow Stem Cells (BMSC)

The post natal bone marrow has been traditionally described to contain two cell types-

1. Hematopoietic stem cells

2. Bone marrow stromal cells

The former is responsible for the production of all forms of blood cells and the latter is a supporting stromal cell capable of generating bone, cartilage, fat and connective tissues. The bone marrow stromal cell line represents an important paradigm of post natal source of multipotent cell line for potential therapeutic use.

The bone marrow is unique being the only organ in which two separate and distinct stem cells and dependent tissue system not only coexist but functionally cooperate. This also makes bone marrow an easy source with plenty of stem cells[6].

Adult stem cells display *transgermal plasticity,* that is, the ability to differentiate into cell types phenotypically unrelated to the cell of their tissue of origin (cartilage, a mesoderm and neural cell, an ectoderm). This property is the basis of the whole concept of the potential use of mesenchymal stem cells in therapeutic intervention.

3.3.1 Heterogeneity of BMSC population

The heterogenous nature of bone marrow cells can be immediately seen upon microscopic examination and on culture colonies. Within the group of stromal cell population there are different cell morphologies. It was noticed that on transplantation to host animal, these cells form ectopic

ossicles, supportive stroma for myelopoiesis, adipocytes and even cartilage.

To date the isolation of a 'pure' population of multipotent marrow stem cells remains elusive. However, stroma cells can be identified using monoclonal antibody, Stro-1, highly expressed by stromal cells are clonogenic. Few cells in the body also express Stro-1, fibroblast, myofibroblast, endothelial cells and hematopoietic cells which mean that stromal cells share the property of these Stro-1^{+} cells. BMSC cells expressed markers for matrix protein of fibroblast, alpha-smooth muscle actin (α-SMA) of myofibroblast, endoglin and MUC-18 of endothelial cells. Thus BMSC is involved in angiogenesis and formation of the supporting stromal tissue as well.

3.3.2 Stem cell fusion

Stem cells of different origin can develop cells of different lineage when cultured together, this is possible through a phenomenon is called cell fusion[7]. Bone marrow cells can fuse spontaneously with other cells and subsequently adopt the phenotype of the recipient cells. Bone marrow cells of male mouse was cultured with embryonic female mouse and later developed embryonic cells with the genetic material of the male mouse.

This phenomenon was also noted in other stem cells within the same animal.

3.4.1 Stem cells in the Gut

Stem cells in the intestinal epithelia lie deep in the crypts giving rise to four cell lineages- absorptive *columnar* cells (*enterocytes* in the small bowel, *colonocytes* in the large bowel), *mucin secreting cell* (goblet cells in the small intestine and colon, gastric foveolar mucous producing cells), *Paneth cells,* which contain large apical secretory granules and *enteroendocrine cells*.

Intestinal epithelia undergoes complete self renewal every 2 to 7 days which increases following tissue damage. This complex heirarchial arrangement of proliferation and differentiation is regulated by the multipotent gastrointestinal stem cells. Stem cells in most tissues resides within *niches* which provide and maintain an optimal microenvironment for stem cell function. In the small intestines stem cells are located in the crypt base just superior to the Paneth cells, in the colon they are located in the mid crypt in the ascending colon and in the crypt base in the descending colon[8].

3.4.2 Molecular markers of GI Stem cells

There are no definite marker to identify gastrointestinal stem cell. Musashi-1 (Msi-1), a neural RNA binding protein, has been found to be elevated in cells just superior to Paneth cells, a postulated stem cell region, and found to positively regulate Hes-1 transcription. Hes-1, a transcriptional repressor first described in CNS, is essential for stem cells self renewal and suppresses differentiation into lineages[9].

3.4.3 Bone Marrow Stem cells Plasticity in the Gastrointestinal tract

The haematopoietic bone marrow stem cell, of mesodermal origin, when transplanted into lethally irradiated animals and humans, as in clinical BM transplant, colonizes host tissues forming new erythroid, granulocute and lymphoid lineages[10]. Bone marrow cells demonstrate plasticity and can also transdifferentiate into hepatocytes, cardiomyocytes, neuronal cells and even intestinal pericryptal myofibroblasts[11].

BMSC from male mouse donor transplanted to lethally irradiated female and in patients with graft versus host disease from male donors were shown to contribute to a population of the regeneration of intestinal subepithelial myofibroblast (ISEMFs) in damaged or injured epithelium. This was observed ***as early as one week post transplant*** and by six weeks post transplant almost 60% of all ISEMFs were of donor origin.[12]

Transplanted BMSC also transdifferentiate to form fibroblasts and smooth muscles in the lamina propria and mucosa. Perivascular and endothelial cell lineage derived from transplanted cells were also observed in new vessels, outlining the potential role of BMSC in vaculogenesis.

The role of fusion in the intestinal epithelial regeneration, where donor stem cells fused with indigenous cells to form diploid cells, is doubtful. The event of spontaneous fusion in vivo is a rare phenomenon (2-11 hybrid clones were formed per 10^6 BMSC [6]).

3.4.4 Pathway of cellular differentiation in the gastrointestinal tract

The molecular pathways that regulate proliferation and differentiation of cells provide a clearer insight into the understanding of the mechanism of tissue regeneration and even neoplastic transformation. The following are the established pathways in tissue regeneration:

The Wnt/β-catenin signaling pathway-

The signaling protein, Wnt, of which there are 19 identified in humans, indirectly inhibit GSK3β resulting in the accumulation of cytosolic β-Catenin.[13] β-Catenin then translocate to the nucleus and interact with members of the Tcf/LEF (T cell factor/lymphocyte enhancer factor) family of DNA binding protein causing activation of the downstream target gene which increases cell proliferation.

The Tcf/ LEF DNA binding protein family-

There are four known members of this family of transcription factors- Tcf-1, LEF1, Tcf-3, Tcf-4. Tcf-4 is expressed in high level in developing intestines and in adult epithelium of colonic carcinomas. Tcf-4 is essential in establishing the stem cell population within the niche of small intestinal crypts. The cryptal niche is activated by the wnt signal pathway from the underlying mesenchymal cells.[14]

Cdx-1 and Cdx-2 homebox genes-

Cdx-1 is expressed throughout the proliferating compartment of the developing intestinal epithelium and adult epithelium. Tcf-4 knock out mouse does not express Cdx-1 in the intestinal epithelium, implying Cdx-1 is a direct downstream target of the Tcf/ β-Catenin complex in the Wnt pathway and is employed in the development of the epithelial niche.[15] Cdx-2 is also expressed in all epithelial cell nuclei in the upper region of the crypt of the descending colon to the rectum. A deceased expression of Cdx-2 is associated with increased dysplasia in these cells.

The forkhead family of transcription factors-

The forkhead, or winged helix[16] produces the Fox proteins and expressed by the gastrointestinal epithelium, is responsible for activation of the Wnt pathway. A mutant Fox-1 has increased increased levels of heparin

sulphate proteoglycans (HSPG) causing over activation of the Wnt/ β-Catenin pathwayleading to increased epithelial proliferation.[17]

TGF- β abd the Smad signaling pathway-

The transforming growth factor β (TGF-β) family are responsible for the inhibition of the epithelial proliferation. TGF- β form a multimeric complex with serine-threonine TGF- β type I and type II (TβRII) receptors causing phosphorylation of Smad2 and Smad3 cytoplasmic proteins and then forma heteromeric complex with Smad4. This complex then translocate to the nucleus and generate TGF- β target gene transcription. [18]

Regulation of stem cell fate and patterning in gastrointestinal crypts and villi-

The Notch signaling pathway support the Unitarian hypothesis that an intestinal stem cells give rise to the four specialized intestinal epithelial lineages. The notch protein regulate the expression of Math1 gene and Hes1 which lead to the differentiation of intestinal stem cells into the intestinal epithelia.[19]

The sonic hedgehog pathway of gastric epithelial cell differentiation-

The sonic hedgehog (Shh) gene encodes a morphogenetic signaling protien for the gastrointestinal tract development, the gastric gland in particular. The Shh gene expression and the GATA-4 transcription factor interaction

regulate the gastric epithelial proliferation through transcription targets, Fox2a, BMP4 and Islet factor 1 (Isl-1).[20]

3.5 Wound healing

An acute wound is defined as a traumatic loss of normal structure and function to recently uninjured tissue after a noxious insult. Wound healing is critical to the survival of the organism which is a highly regulated process of cellular, humoral and molecular events involving in the repair or regeneration of the injured tissue. Classically the phases of healing are

- Haemostasis
- Inflammation
- Cell migration and proliferation
- Scar remodeling

3.5.1 Haemostasis-

The first phase of wound healing is securing haemostasis which is accomplished by vasoconstriction, platelet aggregation and the fibrin deposit from the coagulatin cascades resulting in the clot formation (a fibrin mesh and aggregates of platelets and trapped blood cells).

3.5.2 Inflammation-

The inflammatory response is proportional to the extent of injury and it begins few hours after the onset of injury. There is vasodilatation , capillary leak and migration of inflammatory cells.

The transition from vasoconstriction to *vasodilatation* is mediated by various factors- endothelial products and mast-cell derivatives such as prostaglandins, leukotrienes,histamines and kinins (released by plasma protein-binding molecules via activation of kallikrein).

Capillary leak is primarily mediated by the above factors as well along with the additional influence of complement factors, C3a and C5a.

Leukocyte migration is stimulated by complement factor, collagen, elastin breakdown products, tumour necrosis factor (TNF-α), TGF-β, interleukin-1,PDGF, leukotriene B4 and platelet factor IV.

3.5.3 Cell migration, proliferation and regeneration.

The cellular regeneration and proliferation is the backbone of a successful wound healing. Cell proliferation is stimulated by cell injury, death or mechanical deformation of tissues. The cell growth cycle consists of the G_1 (presynthetic), S (DNA synthesis), G_2 (premitotic and M (mitotic) phases. Most tissues of the body contain a combination of continuously dividing cells or *labile* cells, *quiescent* or stable cells that occasionally enter into the

cell cycle and *nondividing* cells. Cell replication is controlled mainly by the factors in the microenvironment which stimulate or inhibit cell proliferation. The most important factors are those that recruit resting or quiesent cells into the cell cycle.

The columnar epithelium of the gastrointestinal tract contains a greater proportion of labile cells that follow the cell cycle from one mitosis to the next and continue to proliferate throughout life. The connective tissue and mesenchymal cells are quiescent in adult mammals, but proliferate in response to injury. Fibroblasts in particular proliferate widely and constitute the connective tissue response to inflammation.

Control of normal cell growth

Molecular events in cell growth are complex involving an array of intercellular pathways. Aberrations in such pathways may underlie the uncontrolled growth in cancer as well as abnormal cellular responses to disease processes. There are three schemes of intercellular signaling – *autocrine* (cells responding to substances they secrete themselves), *paracrine* (cells responding to substances produced in close proximity) and *endocrine* (cells responding to hormones secreted at a distant site).

Certain growth factors induce cell proliferation by affecting the expression of genes controlling normal growth and are called *protooncogenes*.

Aberrations in the structure of these protooncogenes convert them into *oncogenes* which lead to the uncontrolled cell growth that occurs in cancer. The processes of normal and abnormal cell division thus follow the same pathways.

Cell surface receptors

Cell growth is initiated by the binding of a signaling agent or *ligand*, usually a growth factor, to a specific receptor protein. The receptor ligand complex initiates a specific cellular response. There are three major classes of cell surface receptors.

Receptors with intrinsic kinase activity have an extracellular domain for ligand binding, a single tramsmembrane region and a cytosolic domain which can have either tyrosine kinase activity or less commonly serine/threonine. Dimeric growth factors bind two receptors and form stable dimmers. *Dimerisation* is followed by *autophosphorylation* where one receptor molecule phosphorylates the other forming binding sites for a series of cytosolic proteins.[21] Binding of cytosolic proteins to the phosphorylated tyrosine residues on the activated receptor generate a *signal transduction* cascade which commit the cell to enter the S phase of the cell cycle.

Receptors without intrinsic catalytic activity have a cytosolic domain which directly associates with and activates protein tyrosine kinases in the cytosol which in turn phosphorylates the receptor. Receptors for many cytokines fall in this category and this is termed the *cytokine receptor superfamily.*[22]

G protein linked receptors contain seven transmembrane loops and are called *seven spanning receptors.* Ligand binding activates a cytosolic G protein complex that then activates an effector system generating intracellular second messengers.[23]

Signal transduction systems

Extracellular signals that are detected by the cell surface receptors are then converted into intracellular signals which generate specific cellular responses. This process is called signal transduction. Signal transduction systems are arranged as networks of sequential protein kinases. The important systems are the mitogen-activated protein (MAP) kinase, phosphoinositide-3 (PI-3) kinase, inositol lipid (IP_3), cyclic adenosine monophosphate (cAMP), the Janus kinases/signal transducers and activators of transcription (JAK/STAT) signaling system and the stress kinase system.

Transcription factors

The signal information received by the cell surface receptors is transferred by the signal transducers to the nucleus where regulation of gene expression occurs. This is controlled by transcription factors which playa vital role in cell growth. They have a modular structure with domains for DNA binding and transcriptional regulation (regulatory domain). The DNA binding domain binds to DNA by distinct molecular mechanisms (homeodomain, zinc finger).[24] The regulatory domain either increases (activation domain) or decreases (repression domain) transcription. Various protooncogene maybe activated from among the transcription factors, c-myc, p53 and retinoblastoma. Transcription factors are involved in the regulation of the cell cycle itself.

Cell cycle regulation

The passage of cell through specific phases of the cell cycle is regulated by two types of molecular controls – a cascade of *protein phosphorylation* pathways involving *cyclins* , a set of *checkpoints* that monitor completion of events and, if necessary, delay progression to the next phase of the cycle.

Cyclins are a group of proteins that control the entry and progression of cells through the cell cycle. The cyclins (A, B and E) form complexes with specific proteins called *cyclin dependent kinases (CDKs).*[25] These complexes regulate the passage of the cells to the next stage of the cycle. The levels of the kinases peak during specific phases of the cycle and are then degraded rapidly as the cell enters the next phase of the cell cycle[26] by the *ubiquitin-proteasome pathway.*[27] The activity of the CDK complexes is also determined by binding of *CDK inhibitors* such as p21 and p27, as well as other kinases and phosphates.[28]

Checkpoints provide a surveillance mechanism by sensing problems in DNA replication, repair and chromosome segregation.[29] When checkpoints are activated, they send signals to the cell cycle machinery that arrest the cell cycle. An example is the *p53* tumour suppressor gene that is activated in response to DNA damage and inhibits the cell cycle by increasing the expression of the CDK inhibitor, *p21*.

Growth factors

Growth factors are specific polypeptides that influence the mitotic cycle. They may act on a variety of cell types or have specific target locations. They also have effects on cell locomotion, contractility and differentiation that influence wound healing.

(Table 4- Growth factor and cytokines)

Extracellular matrix

Cell multiplication and differentiation occurs in intimate contact with the extracellular matrix (ECM). There is now evidence that the ECM critically influences these cell functions).[30] The ECM is secreted locally and forms a network of macromolecules in the spaces surrounding the cells. Three groups of macromolecules associate to form the ECM.

i. Fibrous structural proteins like collagens and elastins
ii. Adhesive glycoproteins like fibronectin and laminin
iii. A gel of proteoglycans and hyaluronan.

The ECM has many functions([d]).[31] It sequesters water molecules to provide turgor to soft tissues and minerals to provide rigidity to skeletal tissues. It provides a reservoir for growth factors controlling cell proliferation. It also provides a base for cells to migrate, adhere and proliferate directly influencing the form and function of cells. The degradation of the ECM accompanies wound healing as well as tumour invasion.

Collagen

Collagens provide the extracellular framework for all multicellular organisms. They have a triple helix of three polypeptide α chains and about 30 different chains form 14 different collagen types. Types I (80 to 90%),

II and III are fibrillar and are abundant in the interstitium. Types IV, V and VI are nonfibrillar or amorphous and are present in the basement membrane and interstitium. Type I consist of the triple helix synthesized within the fibroblast. The α chains are synthesised on the ribosome and then subjected to enzymatic modifications including hydroxylation of proline and lysine residues. Following modification, the procollagen chains align to form the triple helix. The collagens are then secreted from the cell, during which time procollagen peptidases clip the terminal propeptide chains forming *fibrils.* Specific lysine and hydroxylysine residues are oxidized by *lysine oxidase* resulting in cross-linkages between α chains of adjacent molecules stabilizing the collagen array and contributing to the tensile strength.

Elastic fibres

While tensile strength of the tissues is provided by collagen, the ability to recoil is provided by elastic fibres.[32] These consist of a central core made largely of *elastin*, a 70-kD protein. Similar to collagen, it is rich in glycine, proline and alanine while it contains little hydroxyproline and no hydroxylysine in contrast to collagen. The central elastin core is surrounded by a microfibrillar network mainly consisting of *fibrillin*, a 350-kD glycoprotein. Fibrillin associates either with itself or with other

components of the ECM.[33] This network is the scaffolding on which elastin is laid down.

Adhesive proteins.

The ECM components are linked to one another and to the cells by *adhesive glycoproteins* and *integrins.* The major adhesive glycoproteins are *fibronectin* and *laminin.* Fibronectin is directly involved in attachment, spreading and migration of cells[34] while laminin is the major glycoprotein in the basement membrane.[35]

Integrins

Integrins are the most important cell surface receptors that mediate cellular attachment to the ECM. They are transmembrane glycoproteins consisting of a single β chain with a series of α chains.[36] The extracellular domains of integrins bind laminin, fibronectin and many other components of the ECM by recognizing specific amino acid sequences. This causes a clustering of receptors with formation of *focal adhesions* which are links of the integrins to intracellular cytoskeleton complexes.[37] These integrin-cytoskeleton complexes function as activated receptors and activate components of the intracellular signaling systems. In this way, integrins help in organising the actin cytoskeleton as well as transduction of signals from the ECM to the cell. Integrins also play important roles in cell-cell

interactions , are involved in leukocyte adhesion , extravasation, platelet aggregation, developmental processes and wound healing.

The integrin-cytoskeleton linkage may also be the key to unraveling the *tensegrity hypothesis.*[38] Physical forces of gravity, hemodynamic stresses, and movement play a critical role in tissue development. Yet, little is known about how cells convert these mechanical signals into a chemical response. It has been suggested that the mechanical linkage between integrins and the cytoskeleton system may be the mechanism for the conversion of mechanical forces into biochemical signals[39] and this theory is under investigation.

Other ECM components

The other main components of the ECM are matricellular proteins, hyaluronan and proteoglycans. *Matricellular proteins* are secreted proteins that interact with matrix proteins, cell surface receptors or other molecules that in turn interact with the cell surface. *Hyaluronan* serves as a ligand for core proteins of the ECM as well as a backbone for large proteoglycan complexes.[40] Due to its capability to bind water, it forms a viscous gel that provides turgor to connective tissue as well as serves as a lubricant in the connective tissues like cartilage. It also associates with cell surface receptors that regulate cell proliferation and migration. *Proteoglycans* are a

group of core proteins attached to polysaccharides called *glycosaminoglycans*.[41] Heparin sulphate, chondroitin sulphate and dermatan sulphate belong to this group and have diverse roles in regulating connective tissue permeability, structure as well as cell growth and differentiation.

EXTRACELLULAR PROCESSES OF TISSUE REPAIR

The destruction of tissues causes damage to the cellular network as well as the stromal framework of the tissue. Even in tissues where the cells are capable of regeneration, some repair occurs by the replacement of nonregenerated cells by connective tissue. This noncellular tissue repair has three components.

i. Angiogenesis and epithelization
ii. Fibrosis

Angiogenesis and epithelization

The angiogenic process becomes active from day 2 of wound healing. The formation of blood vessels is accomplished by two processes. The first of these is angiogenesis sprouting from pre-existing vessels, *neovascularisation* where new vessels form capillaries.[42]. The second is *Vasculogenesis* or the *de novo* differentiation of the precursor endothelial

cells which involves the organisation of lakes of *angioblasts* into a primitive vascular network.

This involves a series of steps beginning with the proteolytic degradation of the parent vessel basement membrane and formation of a capillary sprout. Endothelial cells then migrate towards this bud, proliferate and mature with the inhibition of further growth and the formation of capillary tubes. Periendothelial cells like smooth muscle cells are then recruited to provide support to the endothelial tubes. These steps are controlled by interactions between growth factors, vascular cells and the ECM. The most important growth factor in adult tissues undergoing physiological angiogenesis is the family of *Vascular Endothelial Growth Factors (VEGF).* An embryological model[43] has been developed that elucidates the important role of VEGF in promoting angiogenesis, increasing vascular permeability, stimulating endothelial cell migration and proliferation and regulating the expression of other growth factors. The *integrins* and *matricellular proteins* also are involved in the control of the motility and migration of the endothelial cells. Certain proteases like *plasminogen activators* and *matrix metalloproteinases* play an important role in the remodeling that occurs during endothelial invasion as well as in cleaving

extracellular proteins to form fragments like *endostatin* that regulate angiogenesis.

Blood vessels stem cells

It is well established that haematopoietic and endothelial stem cells (HSCs and ESCs respectively) developed in a highly coordinated manner during the embryological stage forming common precursor, the *haemangioblast*.[4445] The development of HSCs to mature haematopoietic end cells is well established whereas those from ESCs are not. But the invovlemnt of HSCs in angiogenesis is obvious as found in post bone marrow transplants, where these circulating endothelial precursors originated both from the existing blood vessels and the transplanted marrow itself with the latter having greater proliferative potential.[46] Some researchers do not agree on the involvement of bone marrow.[47]

The commonly cited endothelial precursors are endothelial outgrowth cells(EOCs), endothelial progenitor cells (EPCs) and circulating endothelial cells (CECs) . CEC's represent the most mature of these precursors and EPC's are primitive CD-133 positive precursors (EPC's also express 'monocytic' CD14 marker.

The local microenvironment plays a critical role in regulating the function of endothelial precursors. The cytokines and chemokines provide stimulus

for the mobilisation from the bone marrow or circulating endothelial precursors to travel to the damaged tissue. These precursors adhere to the damaged or hypoxic endothelium and generate new blood supply. The presence of precursor cells also act as a biomarker for wound healing and can even be of therapeutic potential in wound healing, especially in diabetic, burns and ischaemic ulcers.[48]

Angiostatin, an anti-cancer drug ,produces a significant impairment in colonic anastomosis in experimental animals but healing was promptly restored after discontinuation of the drug.[49]

Fibrosis

Within 24 hours following tissue injury, fibroblasts and vascular endothelial cells begin proliferating to form a specialised tissue called *granulation tissue* that is the hallmark of healing. It is within this tissue that fibrosis begins. There are two processes involved in fibrosis. The initial step is the migration of fibroblasts to the site of injury followed by their proliferation. The second step is the deposition of ECM by the fibroblasts. The migration and proliferation of fibroblasts are triggered by a number of growth factors. These growth factors are derived from platelets, activated endothelium and a variety of inflammatory cells like macrophages, mast cells, eosinophils and lymphocytes. The most

important growth factor in fibrosis is TGF-β. TGF-β is produced by most cells present in granulation tissue and has many different actions. It causes fibroblast migration and proliferation, increased synthesis of collagen and fibronectin, decreased degradation of ECM by metalloproteinases, migration of monocytes and stimulating angiogenesis.

The migration and proliferation of fibroblasts gradually decreases and they begin to synthesise and deposit ECM components, especially fibrillar collagens, on the granulation tissue. This process continues for many weeks depending on the size of the wound. The growth factors regulating fibroblast proliferation now influence collagen formation. The net accumulation of collagen depends on collagen synthesis as well as degradation, both of which occur simultaneously. Vascular regression takes place and the granulation tissue is gradually converted into an avascular scar.

3.5.4 Remodelling

The transformation of granulation tissue to scar tissue involves a number of changes in the ECM that are mainly brought about by the synthesis and degradation of collagen. These two processes that occur simultaneously bring about remodelling of the connective tissue and stable scar formation. The degradation of collagen is brought about by a group of endopeptidases

called *matrix metalloproteinases (MMPs).* Activated MMPs are inhibited by a family of *tissue inhibitors of metalloproteinase (TIMP)* which are produced by most mesenchymal cells. The equilibrium between MMPs and TIMPs is a key factor in tissue remodelling.

The disturbance of the ECM plays a role in the pathogenesis of anastomotic leakage after large bowel surgery. Patients with impaired anastomotic healing exhibit a significantly lower collagen type I/III, higher expression of MMP-1 and MMP-2 in the mucosal layers and of MMP-2 and MMP-9 in the submucosal layer.[50]

3.6 Colonic anastomosis

The wall of the large intestine consist of four layers – mucosa, submucosa, muscularis propria and the serosa. The mucosa consist of the layer of columnar epithelial cells thrown into villi and folds over the lamina propria, within these villi are the niches of intestinal stem cells that regenerate continuously in normal mucosa and during wound healing. The lamina propria is rich in blood supply and lymphatics along with the enteric nervous plexus. Surrounding the lamina propria is a thin layer of muscle, muscularis mucosae.

The submucosal layer is the most important layer as it supports the anastomotic suture and it consist of loose connective tissue and matrix

protein, predominantly type I collagen. This layer is also rich in blood supply and lymphatics. The muscularis propria consists of two layers of muscles – the inner circular and the outer longitudinal layer.

The healing of a colonic anastamosis takes place in a similar pattern as in dermal tissue. The phases of GI healing are inflammation, proliferation, and tissue remodelling. If the mucosa is the only injured layer, it can be reformed by migration and proliferation. Full-thickness injury, as in bowel resection, provokes a fibroblastic response resulting in scar formation. The inflammatory phase begins after the injury and is characterized by cell infiltration and edema formation. Oedema is mainly confined to the submucosa, which is usually a thin layer of loose connective tissue. It lasts until the end of the second week. Granulocytes are the predominant cell type in the first 24 hrs, and macrophages follow 48 hrs after clean surgical injury. Angiogenesis starts as early as 2–3 days after injury. Fibroblasts and mainly smooth muscle cells are recruited and produce matrix proteins, primarily in the submucosa and the subserosa. Fibroblastic responses are also very strong at the serosal aspect. Smooth muscle cells contribute more to absolute collagen formation than fibroblasts. Mucosal resurfacing is rapid in the GI tract and accomplished within 1 wk. If the injury is severe, accompanied by tissue destruction or bacterial spillage, the events will last

longer and will be more pronounced. After 1–2 wks, the epithelial layer is fully reestablished over a granulomatous notch consisting of proliferating smooth muscle cells and fibroblasts. Reconstitution of the submucosal collagenous network is important for epithelial regeneration. During remodeling, the anastomosis becomes thinner; however, the muscularis mucosae and muscularis propria stay disorganized, allowing the repair process to be recognized even 1 year after operation.

Leak rate

The incidence of anastomotic leaks after low rectal anatomosis is between 2 and 15 percent.[5152] The incidence remains unchanged even with a covering diversion stoma or bowel preparation.[53]

The role of locally injected BMSC in wound healing are being explored and its role in colonic anastomosis, as to our knowledge, has not been reported in literature.

4.0 MATERIAL AND METHODS:

4.1 Animal experiment

A clearance from the institutional animal ethics committee was obtained prior to conducting the experimental study.

20 female and 5 male albino rats of the Wistar strain weighing between 150 to 250 gms were used for the experiment. Bone marrow was harvested from the long bones of one male rat and preserved in a media (GIBCO™ Iscove's Modified Dulbecco's Medium (IMDM) (1X) liquid containing L-glutamine, 25 mM HEPES buffer, 3,024 mg/L sodium bicarbonate) for the experiment on 5 to 6 female rats.

The experiment was conducted on female rats so that the present of the donor stem cells could be detected later by identifying Y- chromosomes at the site of anastomosis using Fluorescence in situ hybridization (FISH) probes.

Randomization

Two colonic anastamoses were done on each animal, one proximal and the other distal. The prepared bone marrow suspension was injected into one of the anastomosis and a media alone was injected into the other according to the randomization table .The rats were then sacrificed

Bursting pressure was done on alternate rats and histological examination done on the others. The pathologist was blinded.

Steps of the experiment

Harvesting bone marrow - A male rat was anaesthetized in an ether gas chamber for few seconds followed by intraperitoneal injection of Ketamine, 10mg/kg (50mg/ml), boluses of ketamine were used intraperitoneally(10mg/kg) as and when required. With aseptic precautions the long bone were isolated, (both femurs, both tibia and both humerus) and the marrow was flushed out with 3 to 4 ml of the Iscove media[1]. The media suspension was stored at 4 degree centigrade and the concentration was calculated manually in a microscope.

Laparotomy and anastomosis

A midline laparotomy was done on a female rat after anaesthetizing in ether gas chamber followed by intraperitoneal ketamine. The proximal colon was identified and transected 3cm from the ileocaecal junction, 3cm from peritoneal reflection, an intramural injection with bone marrow suspension or media alone was done (depending on the randomization

[1] GIBCO™ Iscove's Modified Dulbecco's Medium (IMDM) (1X) liquid

Contains L-glutamine, 25 mM HEPES buffer, 3,024 mg/L sodium bicarbonate, but no -thioglycerol or 2-mercaptoethanol

table) using a 26 gauge needle and insulin syringe till a bleb is raised on the serosal surface (Figure 2). The anastomosis was done using a 6-0 polypropylene (Ethicon®) single layer, seromuscular, interrupted anastomosis under a large magnifying lens. The procedure was repeated on the distal colon. Post operatively the animal was injected with 20 ml of normal saline subcutaneously for hydration and was started on usual feeds from the next day.

The first 10 animals were observed for 7 days before sacrificing for histology and bursting pressure. The next 10 animals were sacrificed at day 14.

4.2 Calculation of bursting wall tension

There are two described methods of testing the strength of experimental anastomoses. The first is the identification of breakdown or leakage by radiological methods that has been recently adapted to animal models.[54] The second method is the testing of the mechanical strength of the anastomosis. This can be done in two ways – by applying a longitudinal stretch force or by increasing intraluminal pressure. It has been found that increasing the intraluminal pressure provides a better reflection of the physiological strain than longitudinal stretching.[55,56] It has also been shown

that the bowel wall tension best characterises the forces responsible for the loss of anastomotic continuity.[57]

In this experiment the bursting pressure was calculated by inflating the bowel, across the anastomosis, with normal saline and recorded via transducer to an electronic monitor.

The strength of the anastomosis in this study was measured by the Bursting Wall Tension (BWT) in dyne10^{-3}/cm based on Laplace's law. It was calculated using the Bursting Pressure (BP) and the anastomotic circumference.

$$BWT = \frac{BP \times 1.36 \times \text{anastomotic circumference}}{2\pi}$$

4.3 Histology

The bowel specimen was resected 5mm on either side of the anastomosis and kept in a 10% buffered formalin solution, processed and stained . Hematoxyilin and eosin (H&E) stain was used for staining the section. Tissues were preserved in paraffin blocks.

5.0 Results

Animals were sacrificed at 7 days in the first group and 14 days in the second group. Three animals died during the experiment and during follow up. One died during induction of anaesthesia, one due to

obstruction and one due to leak and peritonitis. The experiment was repeated on a different animal.

5.1 Bursting Wall Tension

Using , Laplace's law, the bursting wall tension (BWT) was calculated for each anastomosis and subjected to statistical analysis using the SPSS software. It was found that there was no significant difference in either the bone marrow anastomotic site or the control, mean bursting pressure of 44.4 dyne 10^{-3} /cm and 47.6 dyne 10^{-3} /cm, respectively (P=0.779, on Independent *Student t-test*). There was no difference in the proximal anastomosis using stem cells; mean for bone marrow anastomosis- 40.83 dyne 10^{-3} /cm and control- 47.128 dyne 10^{-3} /cm (P=0.251, *Mann Whitney test*). There was no difference in the distal anastomosis either; mean for bone marrow anastomosis- 49.01 dyne 10^{-3} /cm and control- 47.02 dyne 10^{-3} /cm (P=0.602, *Mann Whitney test*).

5.2 Histology

In the animals sacfificed at one week, there was more inflammation and ulceration at the site of anastomosis. The anastomotic site was lined by inflammatory tissue and exudates with marked regenerative changes in the adjacent mucosa. Suture material were lined by histiocytes, multinucleated giant cells and fibroblast. Pericolic adipose tissue showed focal infiltrate

of neutrophils, histiocytes and foci of granulomatous inflammation. There was no significant difference in the colonic wall injected with bone marrow cells or control.

In the set of animals , sacrificed at two weeks, the anastomosis showed better wound healing with good apposition of the layers of the intestinal wall. The mucosal lining at the anastomotic site was continuous but showed focus of ulceration lined by inflammatory granulation tissue with acute inflammation. There was less regenerative changes in the adjecent mucosa and less inflammation in the pericolic adipose tissue. There was more inflammatory response in the anastomosis with bone marrow injection than the control; and more fibrosis in the control anastomosis than in the one injected with bone marrow.

6.0 Pictures

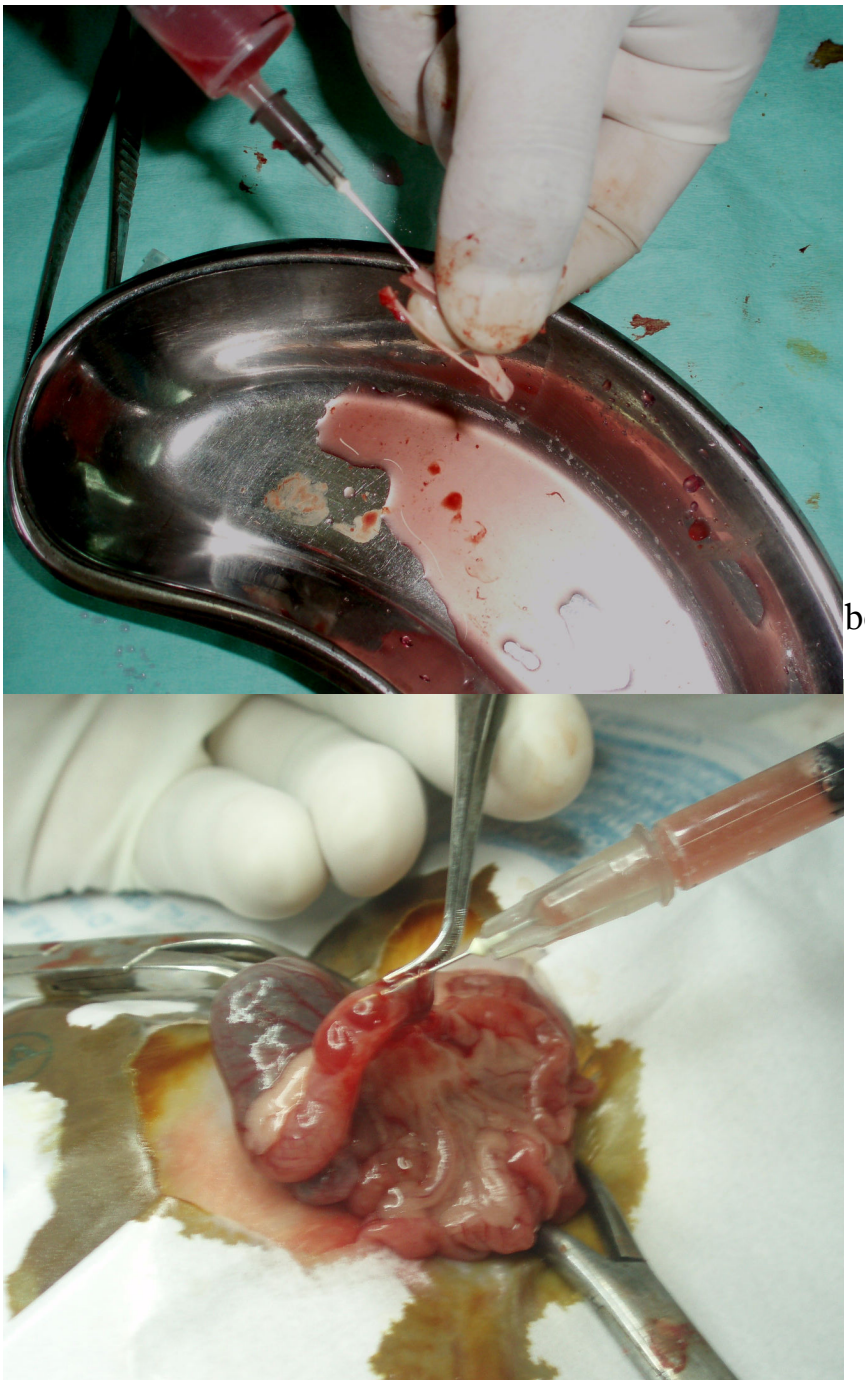
bone

Figure 2- Injecting bone marrow cell suspension into bowel wall

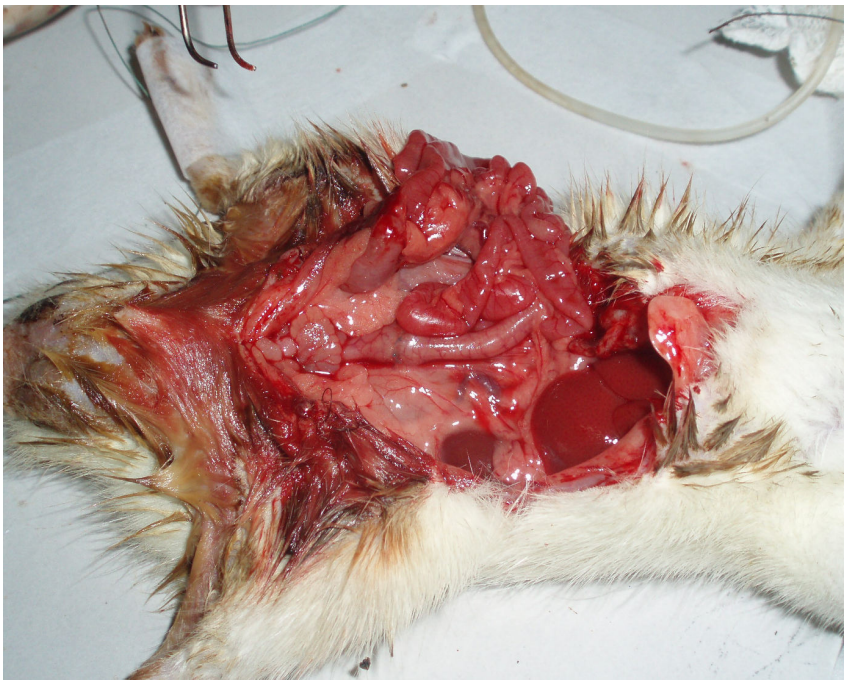

Figure 3- Distal colonic anastomosis

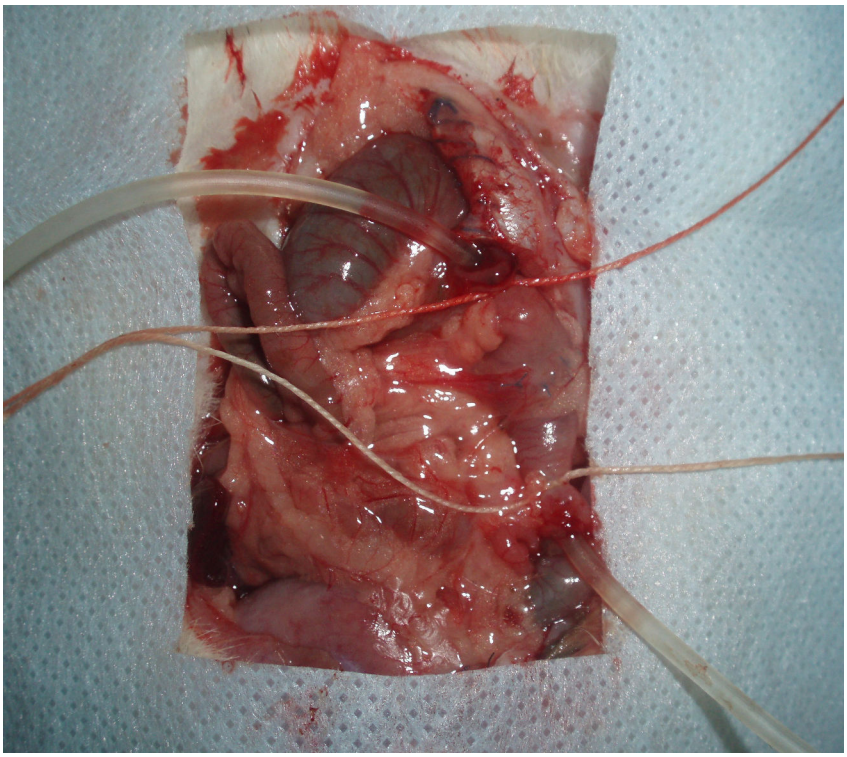

Figure 4- Calculating the bursting pressure

Figure 5- Calculating bursting pressure

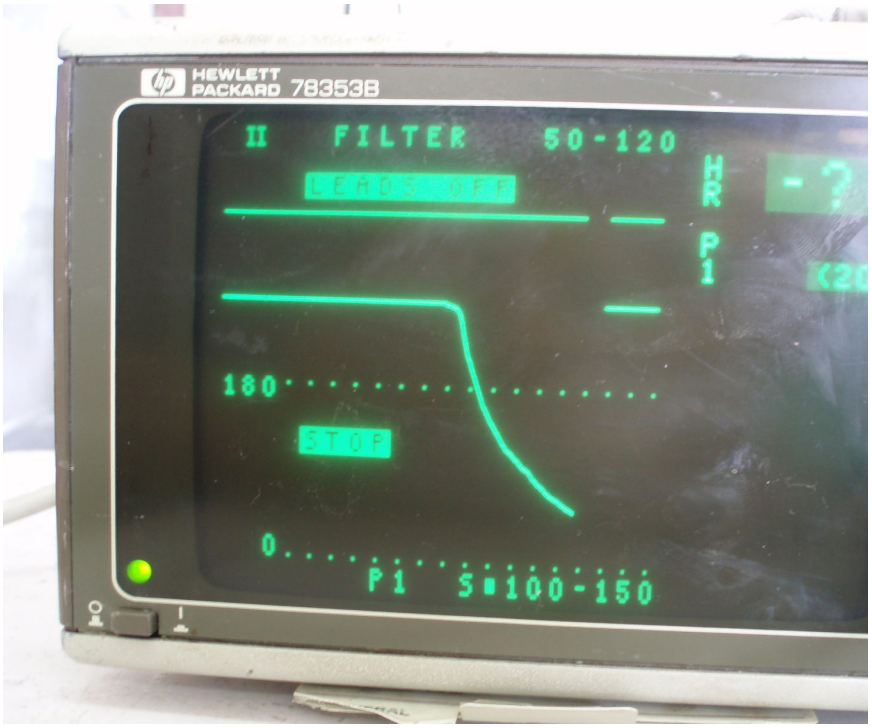

Figure 6- Bursting pressure

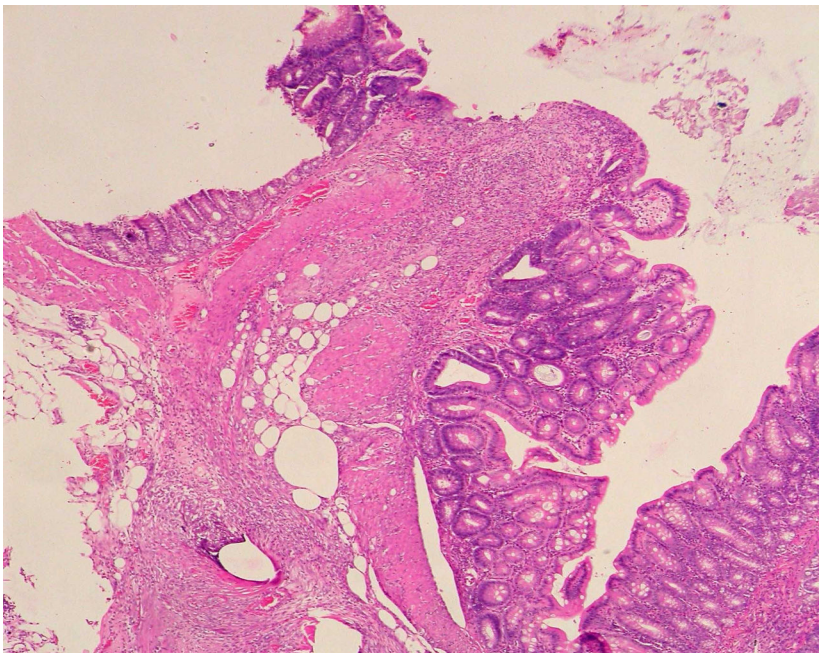

Figure 7- Bone marrow Week 1 (4x), inflammatory cells and exudates at the anastomosis.

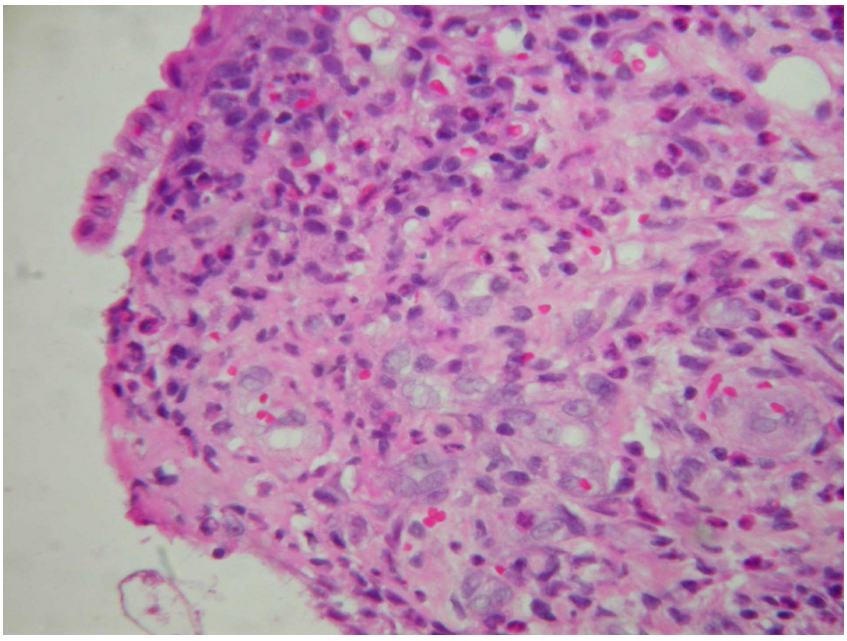

Figure 8- Bone marrow Week 1 (40x), more inflammatory cells at the anastomosis.

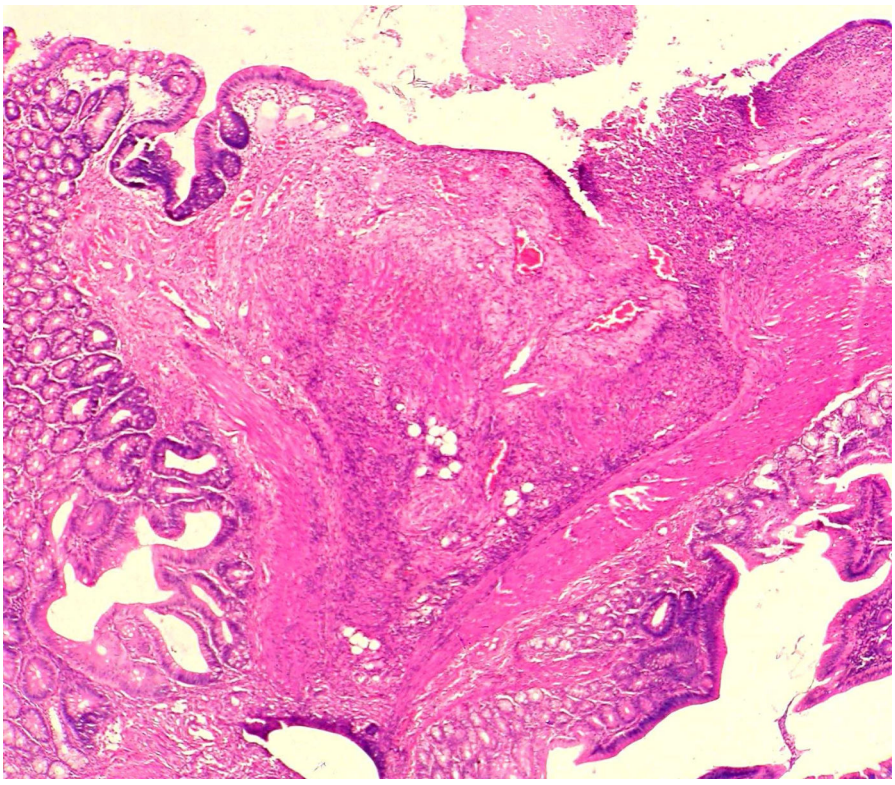

Figure 9- Control Week 1 (4x), inflammation and fibrosis.

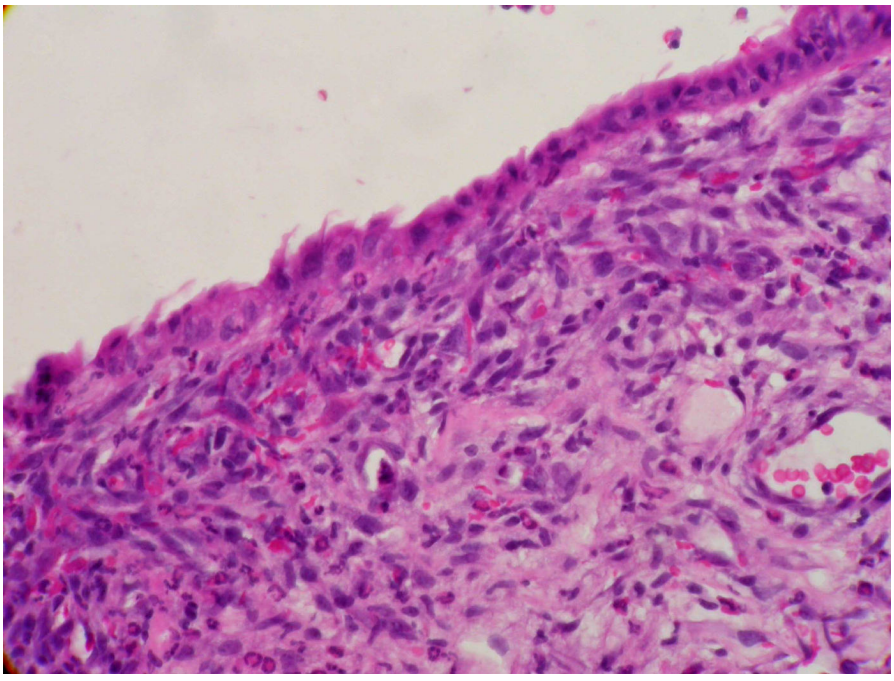

Figure 10- Control Week 1 (40x)

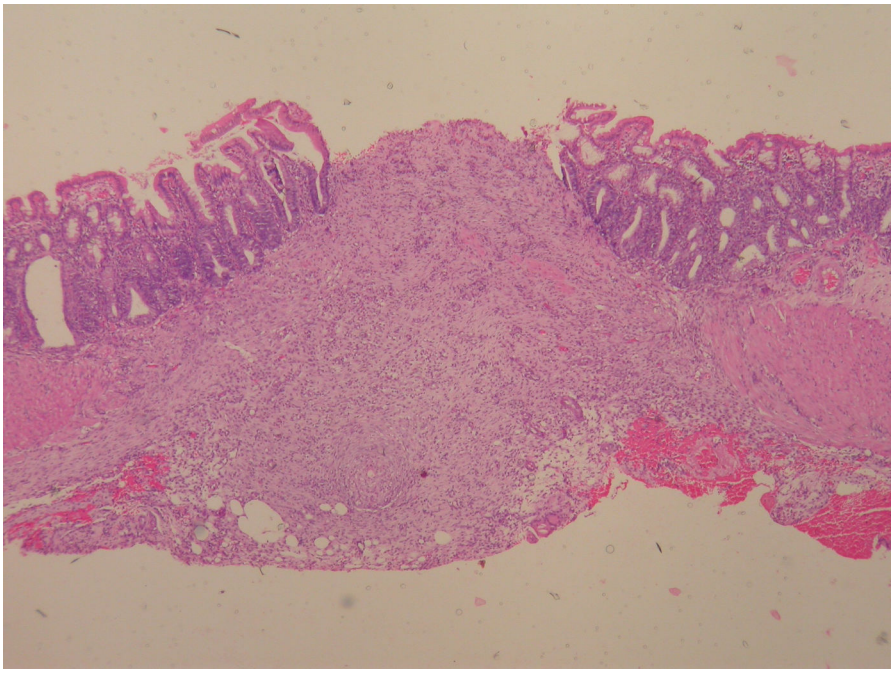

Figure 11- Bone marrow week 2 (4x), fibrosis at the anastomosis but less when compare to the control (below)

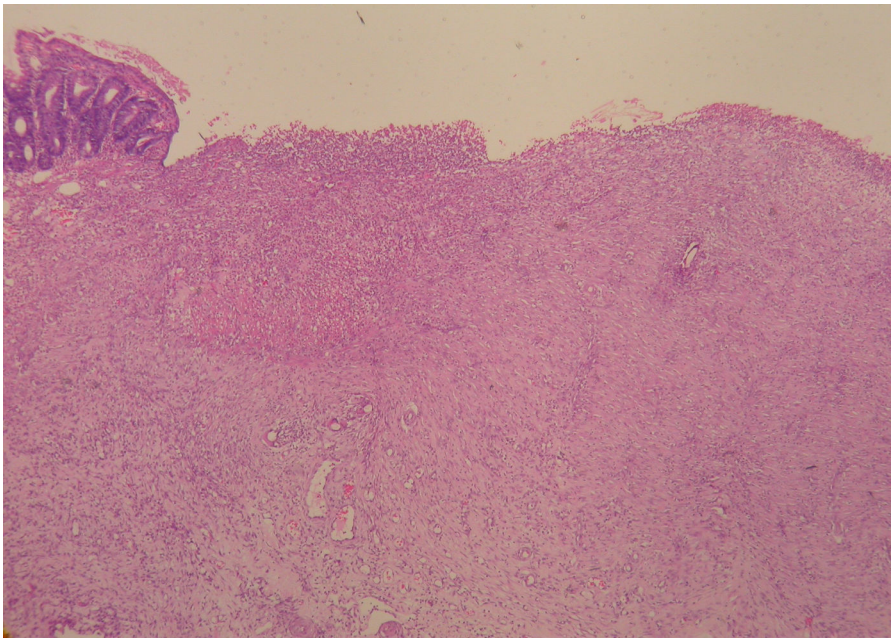

Figure 12- Control week 2 (4x), there was abundant fibrosis at the site of anastomosis.

7.0 Tables

1. Randomization table.

2. Main spread sheet.

3. Histology

4. Growth factor and Cytokines

8.0 DISCUSSION

Colonic anastomosis, is still associated with a high leak rate when compared to the small intestine, it is associated with high morbidity and mortality due delayed presentations and especially when diagnosis is made late or missed. Various techniques and substances were applied (Phenytoin, Nifidepine, surgical glue, etc.) in various studies.

The effect of bone marrow stem cells on the anastomosis in this study does not seem to have a positive influence in the outcome. There was more inflammatory response to the bone marrow cells and less fibrosis. The bone marrow cell may be producing Matrix Metalloprotienases (MMPs) which prevents fibrosis as seen in the control groups.

The colonic vasculature can be a limiting factor for this study, bone marrow suspension injected at the proximal colonic anastomosis can reach the distal anastomosis and vice versa. Thus wound healing maybe similar and producing similar results, both on histology and bursting pressure.

The use of FISH probe in identification of the donor cells would help in consolidating the effectiveness of the use stem cells in this study and or similar studies.

9.0 CONCLUSIONS

i) Stem cells derived from the bone marrow when injected at the site of anastomosis did not significantly alter the bursting pressure or histology at the site of anastamosis.

ii) There was more inflammation when bone marrow derived stem cells were injected thereby indicating an increased risk of leak.

iii) There was less fibrosis in the bone marrow derived stem cell injected group of anastamosis as compared to the control group.

10.0 Clinical Significance and Role for Further Research

This is the first study done using crude stem cells derived from the bone marrow. A study done with cultured stem cells may be more appropriate. Technichal perfection in harvesting and culturing stem cells can be achieved and this may help in future studies.

The use of Fluorescence in situ hybridization (FISH) probe in identification of the donor cells would help in consolidating the effectiveness of the use stem cells in this study and or similar studies.

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ABSTRACT

Title- The Effect of Bone Marrow stem cells in the healing of Colonic Anastomosis in Rats.

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Background and Objectives: Colonic anastomosis have been shown to have high leak rate and is associated with high morbidity and mortality despite advances in technique and technology. The role of bone marrow stem cells in colon anastomotic wound healing is yet to be explored.

Methods: Wistar-albino rats of the same strain were used for the experiment. Bone marrow was harvested from male rats; two anastomosis were conducted on female rats (one proximal and one distal colon). Bone marrow was injected into one of the anastomosis into the intestinal wall and a control was injected into the other anastomosis. The first group of 10 female rats were sacrificed after 7 days and the second group was sacrificed after 14 days. The bursting pressures and bowel wall tension of the anastomosis was determined and histology of the anastomosis was studied.

Results: There was no different in the bursting pressure between the bone marrow injected anastomosis and the control (p- 0.779). The histology of the anastomosis on both arms was similar, but there was less fibrosis on the bone marrow injected anastomosis.

Conclusion: Crude bone marrow does not seem to have a positive influence on the healing of colonic anastomosis in rat model. However the use of culture pure stem cells may help in further studies.