EFFECT OF ESCALATED DOSES OF GAMMA RADIATION IN COMBINED RADIATION AND WOUND INJURY ANIMAL MODEL

DISSERTATION

Submitted by

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DECLARATION

I Varsha Gupta, student of the Thapar Institute of Engineering and Technology, Patiala, hereby declare that the project work entitled, "**EFFECT OF ESCALATED DOSES OF GAMMA RADIATION IN COMBINED RADIATION AND WOUND INJURY ANIMAL MODEL**" carried out from July 15, 2022, to July 15, 2023, is based on your thoughts and ideas. I have faithfully and accurately cited all my sources, including books, journals, handouts, and unpublished manuscripts, as well as any other media, such as the Internet, letters, or significant personal communications. I understand the concept of "plagiarism" and declare that while drafting this dissertation I have refrained from plagiarism. I know that plagiarism not only includes direct copying but also the extensive use of others' ideas without proper referencing or acknowledgment (which includes the proper use of references and quotation marks). If my dissertation is found to be plagiarized at any point in time, I'll be solely responsible and will be ready to accept any decision taken by the competent authority including rejection of my dissertation.

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TABLE OF CONTENTS

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It is certified that the dissertation report entitled "**Effect of Escalated Doses of Gamma Radiation in Combined Radiation and Wound Injury Animal model**" which is being submitted by Ms. VARSHA GUPTA in partial fulfillment of the requirements for the award of the degree of M.Tech. Biotechnology (4thSemester), Thapar Institute of Engineering and Technology, Patiala, is a record of candidate's own work carried out by her from 15th July 2022 to 15th July 2023, under my supervision and guidance. The matter embodied in this report has not been submitted for publication and award of any other degree.

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PLAGIARISM CERTIFICATE

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ABSTRACT

Radiation exposure events on a large scale have always found that victims exposed to radiation are also often suffering from other injuries including blast, burns, or wounds. These types of injuries are known as combined radiation injuries (CRI). CRI is an extremely harmful and deadly form of trauma and multiple studies suggest that conventional trauma and radiation injury have detrimental synergistic effects on the victim. CRIs are known to dramatically increase mortality and delayed wound healing. To study the effect of interventions against combined injuries, the animal model needs to be developed.

In this study, the efforts have been made to develop the CRI model in experimental rats (Sprague Dawley) by exposing the animal to different doses of ionizing radiation (^{60}Co-gamma rays: 5Gy and 6Gy) followed by the creation of dermal wounds (excision wound) on the dorsal skin surface. Animals were then observed for changes in body weight, wound area, and wound score. Further the changes in the hematopoietic system, and inflammation were determined in skin and blood tissues in animals exposed to CRI.

The study showed that with the increase in radiation doses, there was a decrease in survival rate and delayed wound healing. CRI increased the body weight loss, suppressed hematopoiesis, produced systemic inflammation, and damaged bone marrow at various doses (5 Gy and 6 Gy) on days 0, 7, 15, and 30. In conclusion, the results of our study indicated that decrease in survival rate and impaired wound healing was radiation dose-dependent.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Radiation is a form of energy that originates from radioactive elements and travels across space at the speed of light in the form of gamma rays, X-rays, etc. According to these projections, data from the Hiroshima and Nagasaki nuclear blasts and the Chernobyl nuclear accident revealed that more than 60% of radiation victims also experienced a traumatic injury of some sort, most frequently a burn/wound injury. The combination of physical trauma, such as skin wounds, chemicals, and infections, with ionizing radiation exposure is known as combined radiation injury (CRI). It was anticipated that following non-lethal radiation doses, the rate of mortality was increased due to radiation-combined injury. In addition to radiation-combined injury it was also observed that accelerating the body weight loss, exacerbated cytokine and chemokine imbalance, systemic bacterial infection, and disruption of vital organ function, radiation-combined injury reduces survival when compared to radiation alone and can result in multiple organ dysfunction.

The skin is a key organ, which is an exterior defense system that protects the body from the outside environment's temperature variations, pathogens, and radiation and the inflammation may be impacted by radiation either directly or indirectly that develops during radiation therapy rarely goes away in a 24-hour period, causing an accumulating response that increases vascular permeability, vasodilation, and the levels of cytokines and growth factors like transforming growth factors beta, delaying the process of re-epithelialization. The most vulnerable tissue to physical harm is the skin, which causes wounds to occur, and then treated to heal them. The wound healing and infections are significantly harder to manage, radiation damage alone patients often have a higher chance of survival than radiation combination injury patients. The indirect radiation causes DNA damage by interacting with other cell molecules to produce free radicals such as hydrogen and hydroxyl radicals. In addition, exposure to indirect radiation causes a mismatch between cellular pro-oxidants and antioxidants, which results in oxidative stress, decreased endogenous antioxidant defence, tissue damage, and other molecular and cellular effects, It can also result in chromosomal abnormalities, gene mutations in somatic cells, genomic instability, and the suppression of mitosis during the cell cycle.

This study thrives to determine the physiological response to radiation when other injuries are present and also to determine the changes in wound pathology in combination with radiation exposure. Radiation, especially whole-body ionizing radiation (WBI), can lead to acute radiation syndrome (ARS) manifested as extensive gastrointestinal damage, hematopoietic and immunologic suppression. Irradiation combined with wounds decreases body weight, increases the number of bacterial species detected in the tissues, and reduces survival compared to wounds or radiation exposure alone. Radiation induced hematopoietic injury as well as gastrointestinal damage, can decrease the number of red blood cells (RBC), white blood cells (WBC) and platelets (PLT) in the blood circulation. Our research intends to develop combined radiation injury model utilizing Sprague Dawley rats.

In order to achieve this, we looked at how escalated doses of ionizing radiation with static wound size in SD rat affected morbidity and wound healing. Animal models has been developed with different treatment such as animal with radiation only and animal with excision and radiation. To further investigate the biomechanics of wound strength following wound closure, wound area, wound scoring and wound imaging was carried out. Also studied the effect of radiation and wounding on the blood, bone marrow and different organs like kidney, liver, spleen and skin.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The term "radiation" refers to the release of electromagnetic waves and particles as a form of energy. There is no mass to electromagnetic waves since they are vibrations of electrical and magnetic energy. There are a number of suitable mediums in which this energy can be deposited, either partially or completely. Where an adverse effect of radiation is a radiation-induced skin reaction. In most cases, radiation-induced skin reactions are classified as acute or chronic. It is possible that acute radiation-induced skin reactions will cause severe sequelae, affecting the quality of life, prognosis, and cancer treatment progress. In addition to chronic ulcerations and wounds, radiation can also cause fibrosis. There are many factors affecting chronic radiation effects, not just its total dose, such as the type of radiation, the area, the volume, the fraction size,and the schedule of radiation.

SOURCE OF RADIATION: COBALT-60

A radioactive isotope of cobalt called cobalt-60 emits gamma rays.^{60}Cobalt is created when a target material, such as cobalt-59 is bombarded by neutrons. This reaction results from the explosion of nuclear weapons and reactors. To treat various cancers, a teletherapy system emits intense gamma radiation from an externally sealed cobalt-60 source. Cobalt-60 is also used as a tracer in medicine. The neutron in the cobalt-60 atom divides into a proton and an electron in its nucleus, this process is known as beta decay. The atomic number is raised by one as the proton stays in the nucleus while the electron is expelled. The nuclear equation shown below can be used to represent this process

$$^{60}\text{CO} \longrightarrow \text{Ni-60} + \beta^{-} + \text{antineutrino}$$

Dose of radiation: The amount of radiation energy absorbed by an individual or an object is referred to as the dose of radiation. The dose of radiation can be of three types such as:

- **Absorbed dose:** It is used to evaluate the possibility of biochemical alterations in particular tissues.
- **Equivalent dose:** The equivalent dose is used to determine how much biological damage is anticipated from the absorbed dose.
- **Effective dose:** The potential for potential future long-term effects is evaluated using the

effective dose.

Dose rate: Radiation dose rate refers to how much radiation is absorbed per unit of time. A person or object is exposed to specific kinds of radiation at different dose rates depending on the type of radiation, the energy of the radiation, and the distance from the source. The unit of measurement is Gy/min.

Unit of radiation dose

The units for measuring the amount of radiation that has been absorbed by a medium are Rad and Grey (Gy). The radiation absorbed dose is measured in the centimeter-gram-second system of measurement in terms of rad, where one rad is equal to the deposition of 100 ergs of energy per gram of a substance by irradiation. While the unit of radiation is Grey in meter-kilogram-seconds and the SI system of units. One Gy corresponds to one joule of energy deposited in one kilogram of material by IR. Therefore, it can be represented as;

$$1\text{rad} = 100\ \text{ergs/g} = 0.01\text{Gy}$$

$$1\text{Gy}=1\text{J/Kg}=100\text{rad}$$

TYPES OF RADIATION

There are two types of radiation - Ionizing radiation and non-ionizing radiation.

- **Non-Ionizing radiation**

Non-ionizing radiation has enough energy to move atoms in a molecule around or cause them to vibrate, but not enough to remove electrons from atoms. Radio waves, visible light, and microwaves are a few types of this radiation.

- **Ionizing radiation**

There are several sources of ionizing radiation, including X-ray machines, cosmic particles, and radioactive elements. Ionizing radiation is emitted by radioactive elements as their atoms decay. Ionizing radiation is released as a result of radioactive decay. Radon and cosmic rays, which are rays that enter the earth's atmosphere from space, are examples of natural sources of ionizing radiation. It might also come from medical imaging devices like X-ray, CT, or PET scanners. High quantities of ionizing radiation are also released by atomic bombs and nuclear power plant accidents. An individual's body may suffer rapid harm from very high doses of ionizing radiation, including severe skin or tissue damage, acute radiation sickness, and even death. Among the ionizing radiation that is released are alpha, beta, and gamma rays.

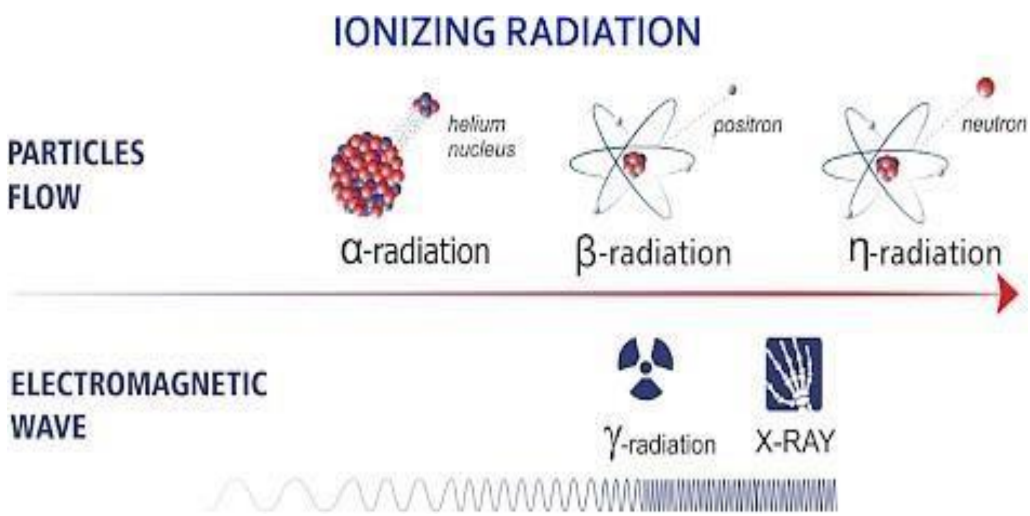


Figure 1: **Ionizing Radiation**

(https://images.collegedunia.com/public/image/8aa01291a7a0e282b88ba24643d7593a.png)

1. Alpha radiation

In alpha radiation, two protons and two neutrons are released by the unstable atom, which is essentially a helium nucleus. The original atom becomes a different element when its protons and neutrons are reduced. Alpha particles interact strongly with matter because of their charge and mass, also it cannot penetrate dead skin cells. It is externally exposed and reaches only the outer layers of human skin and causes severe damage to cells and genetic material inside the body due to high exposure to radiation.

2. Beta radiation

By decaying, an atom gives off a high-speed, negative, or positive charge particle, known as beta radiation. In comparison with the alpha particles, "beta" particles are smaller and more energetic.

In beta radiation, electrons or positrons are emitted from atoms. Since it has a smaller mass, it can travel farther in the air, up to a few meters, and cause tissue damage. Due to beta radiation's weaker affinity for matter absorption than alpha radiation, which results in a greater range, the penetrating strength of beta particles ranges from a few centimeters to meters in the air and from a few millimeters to centimeters in soft tissue and plastic. For example, a sheet of aluminum that is a few millimeters thick can easily shield beta radiation. Beta radiation can harm tissue when it is exposed externally since it can enter the body, but cause less destructive effects. Over a given distance, it loses less energy than alpha radiation, but not by much. Thus, alpha radiation is thought to be more biologically effective than beta radiation.

3. Electromagnetic radiation (X-rays and gamma rays)

High-energy waves like gamma radiation and x-rays can cover huge distances at the speed of light. Both have a high degree of matter penetration. Dense substances, such as bone, tumors, or lead block X-rays. They can therefore be helpful in diagnosing illnesses. Higher-intensity gamma rays have a greater depth of penetration. It has a variety of applications in manufacturing, agriculture, pest management, and more. It can be used to accurately target and remove tumors. A layer of lead several inches thick will stop gamma radiation. Gamma rays are the photon of energy emitted from the unstable nucleus. These rays can completely pass through the animal body and damage tissue, cause injury, and even affect DNA. A very high radiation dose can over a short period of time causes ARS while lower doses can cause radiation-induced cancer.

EFFECTS OF RADIATION

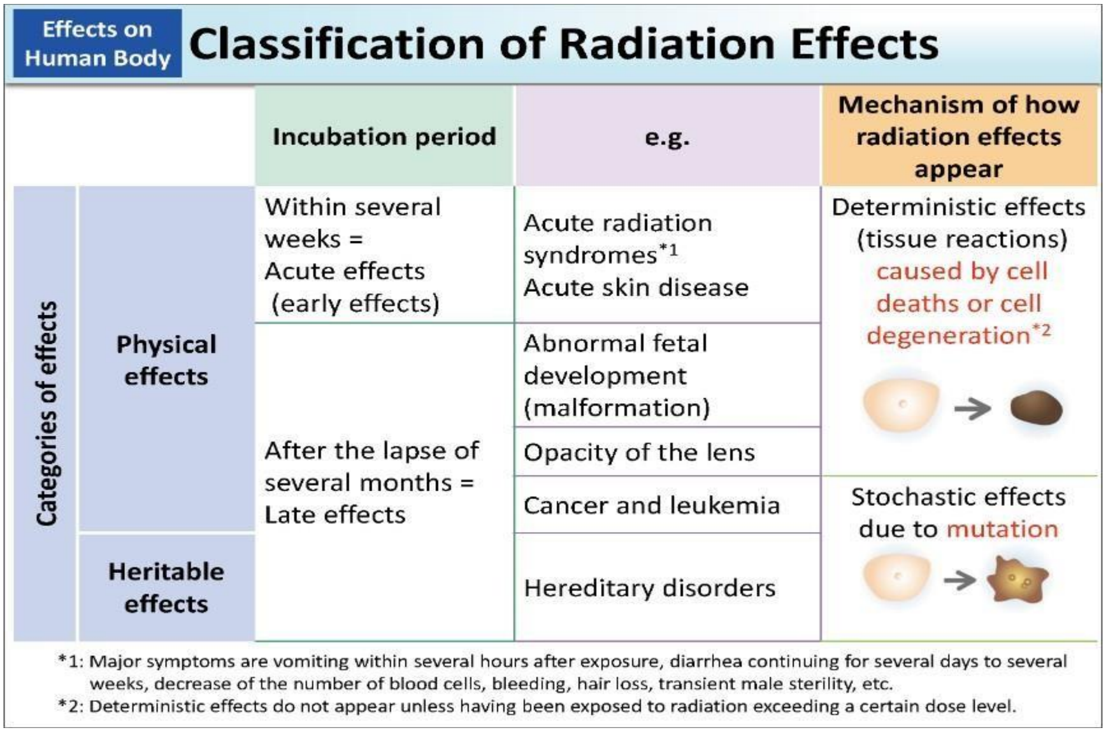
The acute radiation syndrome is characterized by a varietyof signs and symptoms that predominate at different dosage levels and timeframes as a result of damage to various organ systems. The amount of biological damage radiation causes at the cellular level relies on the radiation's ionization density, with more densely ionizing radiation (like -particles) causing more damage per unit absorbed dosage than sparsely ionizing radiation (like gamma rays). High supra-lethal doses of radiation are associated with a neurological syndrome and prompt death within a few minutes to 10 hours (Langham &Brace, 1954). In addition to the neurological symptoms, gastrointestinal symptoms may also be present. Since animals with longer colons would reabsorb more fluids to minimize dehydration and electrolyte imbalance caused by diarrhea. Based on relative histological damage, the small intestine is most likely the part of the digestive system that is most vulnerable to ionizing radiation, also the rapidly proliferating hematopoietic stem cells and progenitor cells of the bone marrow are significantly affected by ionizing radiation. Lymphocytes, the blood cell type that is exposed to ionizing radiation the most, are the first to decline in acute radiation syndrome. So, the loss of lymphocytes, the counts of granulocytes, and platelets will gradually decline and the radiation that causes biological damage and effects is classified into two categories.

- **Deterministic effects**

The effects of radiation-induced cell death are deterministic. It can impair the integrity of organs and tissues, and compromise their function when it occurs at a high enough rate. Thus, increasing the dose leads to greater severity of the effect. In the early stages of tissue reactions, gastrointestinal symptoms may occur, bone marrow failure can occur, skin disturbances may exist, and other symptoms may occur as well. In addition to cataracts, cardiovascular disorders, and necrosis are late tissue reactions.

- **Stochastic effects**

Radiation damage to DNA can occur even at low doses, even with ionizing radiation. So as a result, radiation exposure would result in radiation-induced cancer years later, or inheritable diseases in the descendants of the exposed individual, as well as possible developmental effects depending on the circumstances. Thus, these effects are known as stochastic effects. There are two types of stochastic effect: somatic stochastic effect and genetic stochastic effect. Where somatic stochastic effect are harmful that expose individuals to suffering during their lifetime and in genetic stochastic effect, ionizing radiation damage the genetic material in reproductive cells, and as a result of which these effects are transmitted from generation to generation.



Effects on Human Body — Classification of Radiation Effects

Categories of effects		Incubation period	e.g.	Mechanism of how radiation effects appear
	Physical effects	Within several weeks = Acute effects (early effects)	Acute radiation syndromes*1 Acute skin disease	Deterministic effects (tissue reactions) caused by cell deaths or cell degeneration*2
		After the lapse of several months = Late effects	Abnormal fetal development (malformation)	
			Opacity of the lens	
			Cancer and leukemia	Stochastic effects due to mutation
	Heritable effects		Hereditary disorders	

*1: Major symptoms are vomiting within several hours after exposure, diarrhea continuing for several days to several weeks, decrease of the number of blood cells, bleeding, hair loss, transient male sterility, etc.
*2: Deterministic effects do not appear unless having been exposed to radiation exceeding a certain dose level.

Figure 2: Classification of radiation effects.

(https://www.env.go.jp/en/chemi/rhm/basic-info/1st/03-01-03.html)

HIGH- LINEAR ENERGY TRANSFER (LET) AND LOW-LET RADIATION

The LET of irradiation describes the amount of energy that is deposited per unit of length in the material it passes through, for example, tissue (Danzker, 1959). There are two types of radiation based on how much energy is transferred through matter: high LET and low LET radiation. X-ray and gamma irradiation are characterized by a low LET, inducing sparse and mostly single-strand DNA breaks. In contrast, high-LET α-particles or heavy ions result in very localized DNA damage containing a large amount of double-strand break of DNA(Aten et al., 2004; Eccles et al., 2011; Hagiwara et al., 2017; Timm et al., 2018). The density of ionization events, or LET, is higher for radiation with heavy ions than for photons and protons, giving heavy ions an additional potential biologic advantage because cell death increases with LET. Due to the condensed energy deposition pattern of high linear energy transfer (LET), radiation may lead to its higher lethality than similar doses of low linear energy transfer radiation and also qualitative as well as quantitative differences can occur between high-LET and low-LET effects. However, high LET is more complex and expensive than conventional radiotherapy (Schulz-Ertner, 2007 & Jones, 2009). A normal tissue's level of damage depends on the amount and dose of radiation. It is possible that radiation with high levels of LET may cause a greater induction of cell death than radiation with low levels of LET, but it may also activate different mechanisms. A variety of mechanisms are activated in different organs in response to radiation, resulting in different manifestations of normal tissue toxicity for each organ. As a result of the high-LET radiation, cell cycle-dependent radiosensitivity can be reduced so that cancer cells can be killed regardless of their stage in the cell cycle. Therefore, high LET radiation and low LET radiation have a distinct impact on the induction of DNA damage, causing unambiguous differences in the properties of the damaged DNA (Warters et al., 1978, Goodhead et al., 2006, Jakob et al., 2009)

RADIATION-INDUCED BIOLOGICAL EFFECT

The human body is vulnerable to radiation harm. It develops various symptoms from radiation that affect different organs differently depending on the dose received, exposure, and cell sensitivity. There are various biological effects of radiation such as:

- **Acute Radiation Syndrome**

The acute radiation syndrome (ARS) occurs after whole-body or significant partial-body irradiation (typically at a dose of >1 Gy). ARS can involve the hematopoietic, cutaneous, gastrointestinal, and neurovascular organ systems either individually or in combination. The most rapidly dividing cells are the most sensitive to the acute effects of radiation. Symptoms arising from such exposures are referred to as acute radiation syndrome (ARS). Doses less than 0.5 Gy are not expected to cause acute symptoms, whereas doses of 4.5 Gy are lethal to 50% of exposed persons. The ARS progresses through three phases (Goodhead, 2006).

a) Prodromal phase: 0–2 days after exposure.

b) Latent phase: 2–20 days after exposure.

c) Manifest illness: 21–60 days after exposure.

Acute alterations comprise signs and symptoms that are mostly caused by damage to the skin, CNS, lung, GI tract, and hematological tissues, and they appear within the first two months of exposure.

- **Hematopoietic syndrome**

Ionizing radiation has a profound impact on the rapidly dividing hematopoietic stem cells and progenitor cells of the bone marrow. Ionizing radiation causes a clinically negligible drop in cell counts and a slight, brief stop in cell division at absorbed doses between 20 and 200 rad (0.2 - 2 Gy). Even though some patients may experience temporary health consequences such as slight nausea or headaches after being exposed to a dose as low as 35 rad (0.35 Gy), these symptoms do not qualify as ARS. Clinical ARS is caused by exposures greater than 200 rad (2 Gy) (Donnelly et.al., 2010).

The blood cell line most exposed to ionizing radiation, lymphocytes, are the first to become depleted in ARS. Granulocyte and platelet counts will gradually decrease following lymphocyte reduction. A slower and longer process is used to remove mature red blood cells from circulation. It would be unlikely for acute ionizing radiation exposure to cause rapid onset anemia; underlying causes of red cell depletion (such as hemorrhage) should be investigated instead (Donnelly et.al., 2010).

Symptoms of the hematopoietic syndrome that manifest early on include nausea and vomiting, headaches, exhaustion, fever, and momentary skin reddening. Although none of these problems are brought on by bone marrow radiation exposure, they act as precursors to more severe negative health outcomes. The radiation dose received correlates with their intensity and speed of onset (Donnelly et.al., 2010).

Clinicians should prepare for compromised immunity, which will lead to infection, bleeding, and delayed wound healing due to low platelet counts. 50% decline in the absolute lymphocyte count within the first 24 h after exposure, followed by a further more severe decline within 48 h (Barlow, 1994 & Lebedev, 2001), and the absolute neutrophil count may briefly rise abortively after exposure to doses less than 5 Gy, with the initial neutrophil nadir occurring about a week after exposure.

After being exposed to large doses of ionizing radiation, death usually occurs within a few weeks to months due to illness and/or bleeding. A dose of more than 350 rad (3.5 Gy) will cause the death of almost half of all exposed individuals if they go untreated within 60 days (Donnelly et.al., 2010).

- **Gastrointestinal (GI) syndrome**

After the initial exposure, gastrointestinal symptoms often appear five days later. More severe symptoms develop at doses between 5 and 12 Gy (Chinsoo Cho, 1998) secondary to the loss of intestinal crypt cells and breakdown of the mucosal barrier. These modifications cause cramping stomach pain, diarrhea, and nausea. This first phase is frequently followed by a latent period that lasts 5-7 days and sees a reduction in symptoms. When the digestive system's barrier function is compromised, germs and their toxins flow through the intestinal wall and into the bloodstream. It could be even more damaged by immunosuppression and cytopenia brought on by hematopoietic disease.

At doses between 600 and 1000 rad (6 – 10 Gy), adverse health effects are more severe and abrupt in onset than are the health effects seen following exposures associated with the hematopoietic syndrome. Anorexia, nausea, vomiting (sometimes severe), and crampy stomach pain are typical symptoms that appear one to two hours after exposure. Curiously, vomiting is repressed and not noticed as one of the early symptoms at exposures considerably higher than 1000 rad (10 Gy). Early on in the illness, the occurrence of diarrhea is a particularly worrying indicator. The most frequent causes of death from gastrointestinal syndrome are multisystem organ failure, severe sepsis, and bleeding complications. Within weeks of exposure, patients typically pass away from absorbed dosages of 600 to 1000 rad (6-10 Gy) (or more) (Donnelly et.al., 2010).

- **Neurovascular syndrome**

The nervous system is thought to be the least vulnerable to the effects of ionizing radiation of the three main organ systems included in the spectrum of ARS. Vomiting is suppressed at exposure doses approaching and exceeding 1000 rad (10 Gy), and at the same time, a more general sedative known as the "fatigue syndrome" takes place. Fever and headache are two of the clinical signs of this syndrome, and as the dose is increased, so are abnormal reflexes, dizziness, confusion, ataxia, and loss of consciousness (Donnelly et.al., 2010).

Larger blood arteries are harmed by penetrating ionizing radiation at doses of 3500 rad (35 Gy) and higher, which leads to circulatory collapse. Meningitis, cerebral vasculitis, and elevated intracranial pressure can all occur. Victims who receive dosages greater than 5000 rad (50 Gy) will pass away in 48 hours or less. The anticipated gastrointestinal and bone marrow injuries don't have enough time to show themselves before the sufferer passes away (Donnelly et.al., 2010).

BLOOD

One of the most vital elements of life is blood. Plasma, blood cells, and platelets are the main components of blood, which is a fluid connective tissue. It moves throughout ourbody, supplying different cells and tissues with nutrition and oxygen. 8% of our body weight is made up of it.

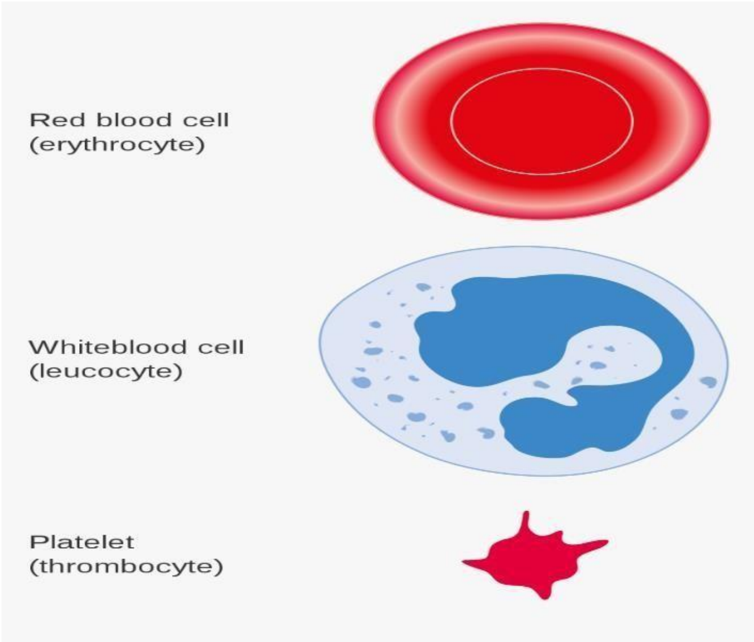


Figure 3: Blood cells

(https://www.pngitem.com/pimgs/m/383-3830965_three-different-types-of-blood-cells-hd-png.png)

Types of blood cells

Blood is made up of cells known as blood components. Each of these cells serves a purpose and performs a specific role in the body. Following are the types of blood cells that circulate throughout the body:

1. Red blood cells
2. White blood cells
3. Platelets

1. **Red blood cells (Erythrocytes):** RBCs are biconcave cells without a nucleus in humans; also known as erythrocytes. The iron-rich protein known as hemoglobin, which is found in RBCs and gives blood its red colour. The majority of blood cells produced in bone marrow are RBCs. They primarily move oxygen from and to different tissues and organs.
2. **White blood cells (Leucocytes):** Blood cells called leucocytes are colourless. They lack colour because there is no hemoglobin in them. They can also be divided into agranulocytes and granulocytes. WBCs primarily support defence mechanisms and immunity.

There are five different types of White blood cells and are classified mainly based on the presence and absence of granules.

- **Granulocytes:** They are leukocytes, and their cytoplasm contains granules. Neutrophils, basophils, and eosinophils are some of the granular cells.
- **Basophil:** basophils contain large cytoplasmic granules, which crucial for creating an immune response that is non-specific to infections and for allergic reactions because they release histamine and dilate blood vessels. Varying between 0.5 and 1% of WBCs. These white blood cells are known as basophils because they can stain when exposed to simple dyes. They secrete serotonin, histamine and heparin.
- **Eosinophil:** Small granulocytes called eosinophil cells, which comprise about 2 to 3% of all WBCs, are formed in the bone marrow. The digestive tract contains significant numbers of these cells. Asthma and allergy-related mechanisms are managed by these cells, along with illnesses caused by parasites that live on vertebrates.
- **Neutrophils:** Neutrophils are typically present in the blood. They are the predominate cells found in pus. Neutrophils with a diameter of 10 to 12 micrometres make up around 60 to 65 percent of WBCs. The cytoplasm contains very small granules, and the nucleus is 2 to 5 lobed. With the aid of lysosomes, neutrophils aid in the eradication of bacteria by acting as a potent oxidant. Only neutral dyes are used to stain neutrophils. So they are known as. Additionally, neutrophils are the first immune system cells to react to an invasion like a virus or bacterium. These WBCs are created daily in the bone marrow and have a lifespan of up to eight hours.

- **Agranulocytes:** They are leukocytes, with the absence of granules in their cytoplasm. Monocytes and lymphocytes are further subclassified as agranulocytes.
- **Monocytes:** Typically, the nucleus of these cells is big and bilobed, measuring 12 to 20 micrometres in diameter. The nucleus typically takes up 6–8% of WBCs and has a half-moon or kidney shape. They are the immune system's garbage trucks. The migration into tissues to remove dead cells, defense against bloodborne pathogens, and swift movement to thelocations of infections in the tissues are the three main roles of monocytes. These white blood cells are known as monocytes because they feature a single bean-shaped nucleus.
- **Lymphocytes:** They are essential for the production of antibodies. Lymphocytes are between 8 and 10 micrometres in size. They're frequently referred to as natural killer cells. They are essential for bodily defense. These white blood cells are colourless lymphocytes because they develop in lymphoid tissue. B lymphocytes and T lymphocytes are the two primary subtypes of lymphocytes. These cells play a crucial role in the immune systems as they are in charge of humoral immunity as well as cell-mediated immunity.

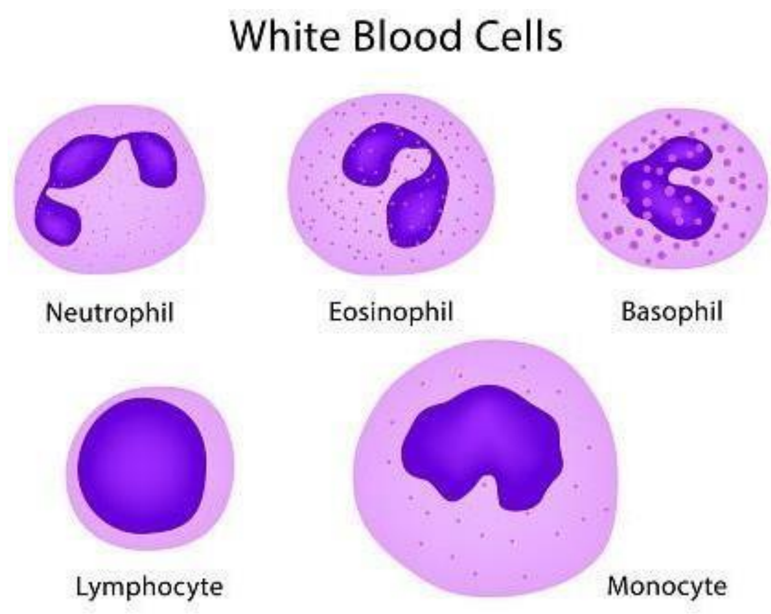


Figure 4: White blood cells (https://media.istockphoto.com/id/1062902018/vector/types-of-white-blood cells.jpg?s=170667a&w=0&k=20&c=d5q7lvRVwW68dyxO8kn37Jb_7g7vt5V98oJxwgjS_8)

3. Platelets (Thrombocytes)

Thrombocytes are specialized blood cells produced from bone marrow. When there is bleeding or hemorrhage, platelets are at work. They aid in the coagulation and clotting of blood. When there is a cut or wound, platelets aid in coagulation.

BONE MARROW

A complex microarchitecture is present inside the bony skeleton that supports and aids in movement of the human body. Adipocytes and blood cells (across a wide spectrum of development) coexist in the cavities made by the trabecular architecture of the bones core. The formation of blood cells takes place in this tissue, which is called bone marrow (hematopoiesis) (Munsch et. al., 2020). About 4% of the weight of an adult human is made up of bone marrow (about 2.6 kilograms). Highly vascularized and innervated tissue. Hematopoiesis, oxygen transport, defense against foreign invasion, and hemostasis are among the key roles. Aids in clearing out old cells from circulation. Consists of a non-vascular component as well as a vascular component. Red marrow and yellow marrow are the two primary subtypes of bone marrow tissue (Vaes et.al., 2017).

A vascular section and a non-vascular section are distinguished in bone marrow. Blood arteries in the vascular part of the bone carry blood stem cells and mature blood cells away from the bone and into the bloodstream. The non-vascular region is where blood cell synthesis takes place, or hematopoiesis. White blood cells (macrophages and plasma cells), fat cells, immature blood cells, and fine, branching reticular connective tissue fibers are all present in this region (Alliston et.al., 2018).

Depending on how much fat it contains, normal bone marrow is separated into red and yellow marrow. Red marrow is made up of two additional stem cell subtypes, myeloid and lymphoid, are produced by hematopoietic stem cells. Red blood cells, white blood cells, or platelets are formed from these cells. Reticulum supporting the stroma (phagocytes and undifferentiated progenitor cells) isolated fat cells rich vein supply mostly restricted to skeletal system bones, such as those in the skull, pelvis, spine, ribs, sternum, shoulder blades, and close to the attachment points of the long bones in the arms and legs. Red marrow produces blood cells in addition to assisting other organs, such as the spleen and liver, in removing outdated cells from circulation (Alliston et.al., 2018).

The components of yellow marrow are identical to those of red marrow, with the exception that fat cells make up the vast majority (80% vs. 40%). inadequate vascular supply constituted of dormant hematopoietic tissue. found in long bones shafts and spongy bones. Yellow marrow can be changed into red marrow in order to create more blood cells when the blood supply is critically low (Alliston et.al., 2018).

Structure of bone marrow

Bone marrow is made up of hematopoietic tissue islands and adipose cells that are bordered by vascular sinuses and interspersed inside a trabecular bone meshwork.

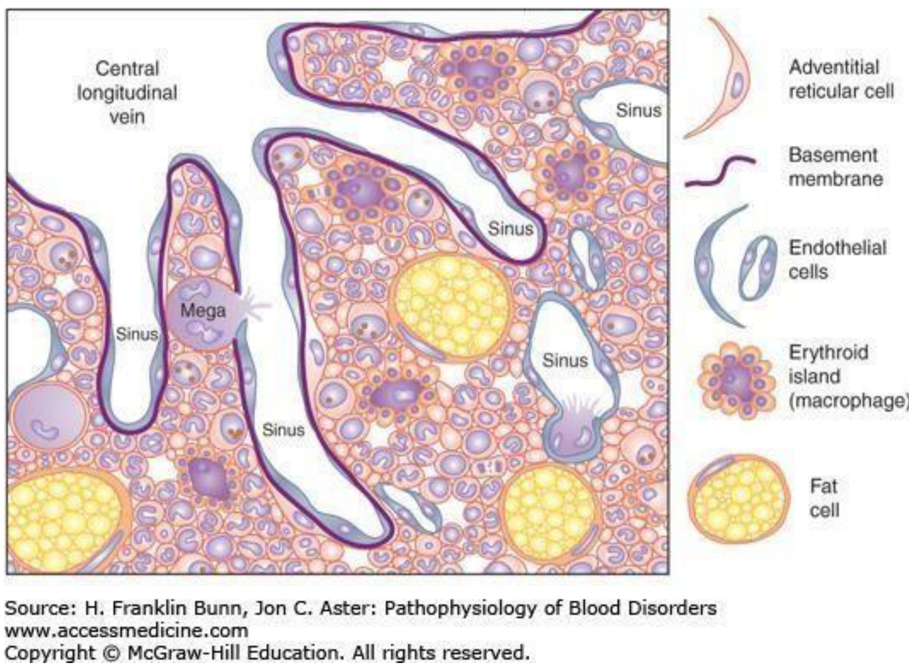


Figure 5: Structure of bone marrow

(https://microbenotes.com/wp-content/uploads/2018/06/Structure-of-Bone-Marrow.png)

The bone marrow can be structurally divided into vascular and non-vascular sections, and it is made up of both cellular and non-cellular components. Hemopoietic cells of diverse lineages and maturities are interspersed amongst fat cells, collagen fibers, fibroblasts, and dendritic cells in the bone marrow's non-vascular region. The vascular part of the bone contains blood arteries that carry produced mature blood cells and blood stem cells out of the bone and into circulation, where hematopoiesis occurs.

Effects of damage in bone marrow

High doses of ionizing radiation exposure cause bone marrow failure, which ultimately results in death. As a result of the abnormally high amount of functioning blood cells, sub-lethal doses of radiation will result in bone marrow suppression, a less severe form of bone marrow failure, immunosuppression, and can damage the cells in the bone marrow. The RBC, WBC, or platelets decrease following radiation exposure when the hematopoietic stem cells in the bone marrow are harmed, the weight loss of lymphoid organs, and the decrease in erythrocytes in the blood after irradiation. Low energy is the primary symptom of anaemia, which is brought on by insufficient red blood cell formation. Infection risk is increased when white blood cell levels are low.

COMBINED RADIATION INJURY

The term combined injury refers to radiation injuries, as well as tissue injuries caused by blasts and thermal energy released by detonations of radiation dispersal devices or nuclear weapons, there is a significant gap between identifying, obtaining, and implementing efficient medical care strategies. According to estimates based on the atomic bombings of Hiroshima and Nagasaki and the Chernobyl nuclear accident RCI might account for up to 65% of all injuries noticed after an air burst nuclear detonation and after studies of the somatic and genetic effects of the atomic bomb exposed radiation in the 1940s, Although it is impossible to assess the exact burden of morbidity and mortality attributable to certain causes, analysis of historical data reveals that up to 40% of survivors may experience radiation and burn injuries.

Radiation injuries with wound injuries, in the absence of burns, would comprise 5% of the expected combination injuries, with the remaining 35% of injuries attributed to other causes (Geiger 1964, Conklin et al. 1983). The explosion of a radiological dispersal device or attacks on nuclear power plants or nuclear waste storage sites are only a few examples of these attacks. It is the worst-case scenario would be the detonation of a nuclear bomb, which would produce vast amounts of radioactive fallout, a powerful burst of immediate radiation, and enormous amounts of blast and heat damage. Additionally, it might leave thousands or even hundreds of thousands of injured survivors. So, bythis exposure to radiation with injury, several animal models have shown that there is an increase in mortality. There are several factors that influence the effects of radiation, including overall dose, dose rate, radiation quality, and the fraction of the body that is exposed to radiation, which is more harmful to irradiate the body in larger amounts and to a larger fraction of the body at higher doses. As a result of combined injury, the body is left with acute myelosuppression, impaired immunity, fluid imbalances, metabolic disorders, massive cellular damage, and disruption of vital organ functions, leading to multiple-organ dysfunction syndrome, the most frequent causes of death after combined injury (Alpine, 1954 & Akashi, 2005). So, by the combined radiation injury 10% of the 237 radiation-exposed casualties after the Chernobyl reactor disaster had thermal burns. There have been instances of burns and wounds in animals, such as mice, rats, and dogs, etc., which typically increase mortality following otherwise non- lethal irradiation. In comparison to wounds or radiation exposure alone, irradiation reduces body weight, increases the variety of germs found in tissues, and lowers lifespan in mice. Here, we describe modifications in molecular and cellular biomarkers in rats that skin-wound damage after receiving 60Co-irradiation to mimic a classic combination injury.

Whereas, wounds are injuries that cause the skin or other body tissues to splinter, for Example Cuts, scrapes, and scratches. Wounds are commonly caused by an accident, but surgery, sutures, and stitches can also create them. There are variations were found in the dimensions of the wounds. Out of the 55 reviewed articles, only four studies (7.27 %) used the same size wound in the same location with the same wounding technique, which was an incisional wound on the dorsum of the rats (Sequeira et al., 2000; Capon et al., 2001; Trabucchi et al., 2002; Jakob et al., 2009). It is also defined as a break or damage in the epithelial integrity of skin, resulting in anatomical, physiological, and functional disturbances of tissue, with or without damaging the underlying connective tissue (Ramzi et al., 1994 and Strodtbeck, 2001). The skin is the body’s first line of defense the surface of which is protected by a thin, acid film produced by the sebaceous glands called the acid mantle. This acid mantle is a dynamic barrier that regulates the skin's pH and maintains microorganisms called the normal flora that help prevent pathogens from entering the body. Pathogens will often displace some of the normal flora and colonize certain locations, but most of the time this does not lead to infection and does not stimulate an immune response. However, when the skin is broken or if the immune system becomes compromised, any of the microorganisms colonizing the skin or introduced to the wound can cause an infection. An infected wound is a localized defect or excavation of the skin or underlying soft tissue in which pathogenic organisms have invaded into viable tissue surrounding the wound.

POSSIBLE MECHANISM OF RADIATION INJURY

Oxidative stress

Due to combined radiation injury, some free radicals are generated through cells in the body by irradiation, which develops some effects, and by these effects, the roles of the mitochondria change noticeably. Numerous cell-based targets ofnitric oxide (NO) exist. whereas inducible nitric oxide synthase is the primary source of NO (nitric oxide) during stressful circumstances like inflammation, which is crucial to the process of oxidative stress. This effect may lead to an increase in superoxide generation, which amplifies the Oxidative stress (Aktan, 2004). Whereas, water makes up the majority of a cell's mass, accounting for roughly 70–80% of it. The indirect cellular harm is caused by the water's radiolysis by-products. An electron is released from the water molecule when a charged particle interacts with it, creating an ionized water molecule (H_2O+).

$$H_2O \longrightarrow H_2O + e^-$$

The highly reactive hydroxyl radical, which is created when the ionized water reacts withanother water molecule. A free radical is a chemical compound that has an unpaired electron in its outer orbit shell and is extremely reactive and electrical neutral.

$$H_2O+H_2O \longrightarrow H^+ + OH\bullet$$

In order to form an aqueous electron (H2O-), the unpaired electron produced by the ionization of the water molecule must first recombine with the water ion or, more likely, be taken up by another water molecule. In the presence of water, this aqueous electron will most likely break down into a hydroxyl ion (OH-) and a hydroxyl radical (H). Since neither the hydrogen ion (H-) nor the hydroxyl ion (OH-) has an excess of energy. A water molecule will be recreated when they join. Consequently, the final outcome of irradiating water will be:

$$H_2O \longrightarrow H\bullet + OH\bullet$$

The radicals have a lot of energy and are capable of destroying chemical bonds. In addition, these free radicals can react with other common molecules or reunite with one another by sharing an unpaired electron in a chemical link to generate H_2O, H_2, or H_2O_2. At this level, oxygen effects are believed to be present. The hydrogen radical and the peroxy radical can combine because oxygen is a great electron acceptor.

$$H\bullet + O2 \longrightarrow HO2\bullet$$

After being exposed to radiation, there is an instantaneous production of an inflammatory reaction. It plays a role in the development of radiation-induced fibrosis as well. The redox activation is significantly influenced by inflammation. Where, pro-inflammatory cytokines (IL-1, IL-3, IL-5, IL-6, and tumor necrosis factor TNF-alpha) are primarily responsible for the early inflammatory response to radiation. Eosinophils and neutrophils may respond locally to these stimuli, causing tissue damage that is self-perpetuating and the loss of protective barriers. Reduced inflammatory cells in the wound, decreased growth hormones, and decreased collagen synthesis are some of the mechanisms behind radiation combined with injury-impaired wound healing. All resident cells, including as keratinocytes, fibroblasts, and endothelial cells, respond to irradiation in the acute phase by activating early response genes and proteins, which include several growth factors, chemokines, and cytokines. So, the inhibition of proinflammatory cytokine can improve skin tolerance but can reduce the inflammation of the dermis, whereas the skin is the biggest organ of the body, and it is essential in defending the body against threats such as infections, fluid imbalances, mechanical stress, and thermal dysregulation.

ROS- Induced damage to DNA

Excessive free radical synthesis during oxidative stress may change DNA's base sequences, cause DNA backbone breaks, and result in DNA lesions. Base damages, single strand breaks (SSBs), and double strand breaks (DSBs) are a few examples of these lesions. These damages might not, however, be enough to result in cell death. It is generally agreed that SSBs are the more frequent of these, but DSBs are the most damaging and one of the main causes of cell death. SSBs, which differ from DSBs in that they only affect one of the two strands of the DNA molecule, are commonly accompanied by faulty or mismatched 5′- and/or 3′-termini. Changes in the genetic material lead to a range of chromosomal abnormalities. A few of these are:

- **Dicentric chromosomes:** Following replication, two broken chromatids in different chromosomes combine to form dicentric chromosomal abnormalities. It can act as a significant indicator of radiation exposure.
- **Acentric chromosomes:** A chromosome without a centromere is another type of chromosomal abnormality that can occur.

EFFECT ON SKIN

Structure and function of the skin

Skin serves as a barrier that protects the body from physical harm, infections, and fluid loss. It also includes immune-neuroendocrine functions that help to keep the body in a state of homeostasis. In the skin, there are two distinct layers: the epidermis and the dermis. An outermost layer of skin, which is composed primarily of keratinocytes with multilayered epithelium, and the interfollicular epidermis. The basal region of the epidermis is attached to the basement membrane. Associated with the basement membrane is a highly vascular layer of dermis, containing extracellular matrix and fibroblasts. Being the outermost layer of the body, skin is continuously exposed to external factors, sometimes resulting in altered structural and functional status. One such variation is the wound, resulting from a wide range of external factors and certain physiological alterations leading to altered structure and loss of functional competence of the skin and underlying tissue. These wounds disrupt the epidermis and dermis resulting in loss of barrier function.

Skin is one of the most complex organs, comprising more than 5 different types of cells contributing to its anatomical makeup. In various cell types that are present in the epidermis are:
Keratinocytes: are the main cells type present in the epidermis constituting 95% of cells.
Melanocytes: these cells produce pigment and are found in basal layers of the epidermis.
Langerhans cells: they have significance in immunological cells and can be initiated through the mid-dermis.

Merkel cells: these are part of the decarboxylation system and are found in the basal layer present in the epidermis.

Various cell types are found in the dermis:

Fibroblasts: It is a type of cell that contributes to the formation of connective tissue, a fibrous cellular material that supports and connects other tissues or organs in the body.

Macrophages: A type of white blood cell that surrounds and kills microorganisms, removes dead cells, and stimulates the action of other immune system cells.

Mast cells: They are accountable for various immunological reactions and interactions.

The Dermis layer plays an essential role as it attaches to different skin layers also. Change of metabolism in the dermis inspires growth strength of the epidermis, hair follicles, and glands of the skin.

Hypodermis: Hypodermis is the deepest layer of skin. This layer provides interaction between the skin and the underlying tissues in the body such as muscles and bones.

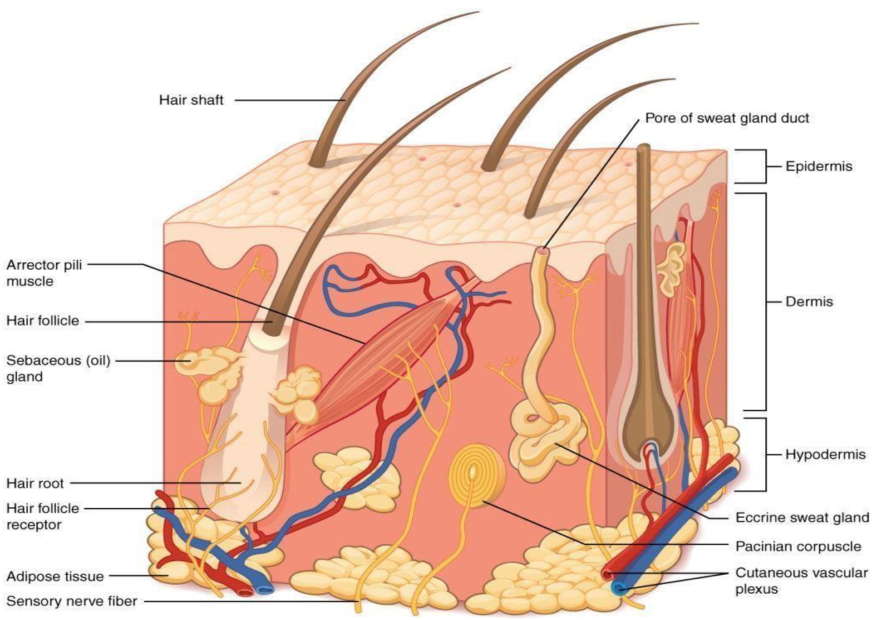


Figure 6: Structure and function of the skin.

(https://s3-us-west-2.amazonaws.com/courses-images/wp-content/uploads/sites/1223/2017/02/08004822/501_Structure_of_the_skin-1024x803.jpg)

Effect of radiation on skin

Radiation exposure can harm the skin's basal cell layer and cause inflammation, erythema, and dry or moist desquamation. Epilation can also result from radiation damage to the hair follicles. The fastest-growing cells, such as those in hair follicles, sebaceous glands, the basal layer of the epidermis, and the targeted tumor, are those that are most affected by radiation. After radiation exposure, the skin on the affected area of the body may become dry and peel, itch (a condition known as pruritus), and turn redder or darker. The skin may develop swelling, wetness, and infection or resemble sunburn. We refer to this as a wet reaction. As damage occurs in the deeper basal layer cells first and takes time to reach the more superficial layers of the skin, radiation-related alterations in the skin are typically not immediately noticeable. In addition to these physical alterations, irradiation has been linked to decreased keratinocyte function, fibroblast migration and proliferation, and disorganized extracellular matrix proteins, particularly collagen in the dermal layer. After exposure the irradiation the number of keratinocytes and fibroblasts was decreased and the matrix in the dermal layer was disorganized.

WOUND

The injury that breaks the skin or other body tissues is called a wound. They consist of skin punctures, scrapes, scratches, and cuts. Wounds are frequently brought on by accidents or the removal of tissue from the body using a scalpel (a sharp knife), laser, or other cutting tool.

Classification of wound

- **Clean wound-** A clean wound is one that was created in sterile circumstances, where no organisms were present and the skin was expected to heal successfully.
- **Contaminated wound-** Usually the result of an accident, a contaminated wound contains infectious organisms and foreign objects.
- **Infected wound-** A wound that is infected shows clinical indications of infection, such as a yellow appearance, discomfort, redness, and pus pouring from the site.
- **Colonized wound-** Colonized wounds are persistent, include harmful organisms, and are challenging to treat.

WOUND HEALING

The human body's ability to provide protection from the outside environment is decreased as the epithelium and connective tissues are damaged. As a result, it is critical to refabricate a functional epidermis or even other layers of skin. This is accomplished through a series of intersecting processes known as wound healing or wound repair. After the injury, skin integrity needs to be quickly recovered to sustain its function. The wound healing process involves cytokines, chemokines, growth factors, extracellular matrix, and regulatory molecules, as well as peripheral blood mononuclear cells, resident skin cells, and extracellular matrix. The events of the wound healing process are categorized into four sequential phases, each often overlapping with the succeeding one. The four distinct phases of wound healing include (a) Hemostasis, (b) Inflammatory, (c) Proliferative, and (d) Remodeling (Stadelmann et al., 1996).

Phases of wound healing

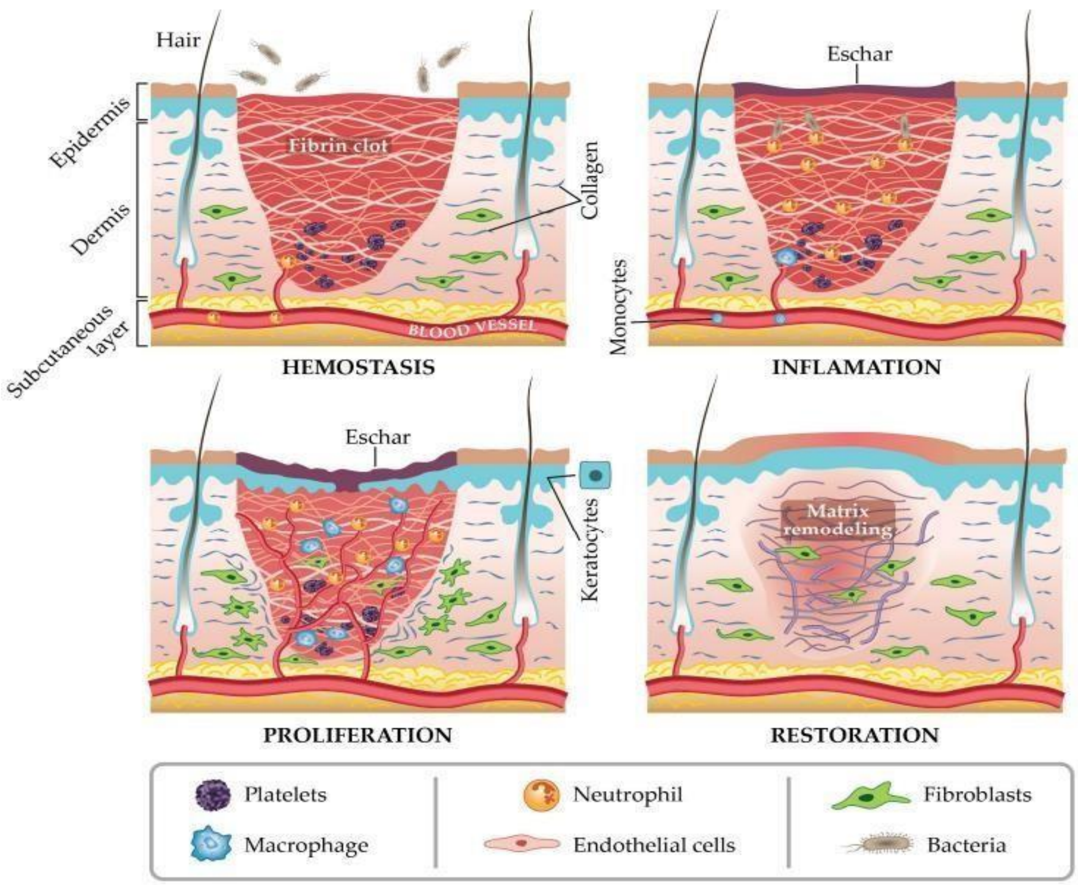


Figure 7: The four stages of wound repair

(https://www.researchgate.net/figure/The-four-stages-of-wound-repair_fig1_327737302)

Phase I: Hemostasis

When the skin is injured, cells are damaged and blood vessels are injured, then immediately after a wound, blood vessels constrict and platelets aggregate, activating complement cascades and clotting. The combined effects of these events lead to hemostatic clot formation. During homeostasis, the clot is an active matrix of proteins and cells that provide temporary protection for the wounds, as well as ensure their healing. In addition to acting as an early mediator of clotting, thrombin is an important component of platelet function. After damage to tissue, fibrinogen from the plasma is converted into an insoluble fibrin clot by thrombin and promotes platelet aggregation. It also stimulates the release of pro-inflammatory cytokines by endothelial cells, such as CCL2, IL-6, and IL-8, which induce the migration of monocytes. These early-produced cytokines are usually proinflammatory, causing monocytes to differentiate into macrophages.

Phase II: Inflammation

The inflammatory Phase is the second stage of wound healing and begins right after the injury when the injured blood vessels leak discharge (made of water, salt, and protein) causing localized swelling. Inflammation controls bleeding and prevents infection. During this phase, damaged cells, pathogens, and bacteria are removed from the wound area. It is only problematic if prolonged or excessive. The chemokines, vasodilation, increase the blood vessel permeability and all help to attract immune cells. In order to coordinate and maintain the wound healing events, immune cells are crucial for the shift from the inflammatory phase to the proliferative phase.

Phase III: Proliferation

The granulation phase of cutaneous wound healing is also known as the proliferative phase, which is characterized by active fibroplasia, epidermal regeneration, and wound contraction. Over the past few years, a great deal of research has been done on the function of fibroblasts in wound healing. Fibroblasts and myofibroblasts are primarily responsible for producing new ECM required for supporting cells and blood vessels, which offer nutrients and oxygen required forcell growth and proliferation. Some fibroblasts are already present in the wound, dispersed throughout the tissue, while others are produced from surrounding undifferentiated mesenchymal cells. When compared to typical dermal fibroblasts, myofibroblasts produce more ECM components. Fibroblasts begin producing collagen 3 to 5 days after injury, and TGF, PDGF, (FGF2), and Insulin-Like Growth Factor 1 (IGF-1) are the primary growth factors that accelerate this process. After ECM production and proliferation, wound healing moves into the remodeling phase, which can extend for days, months, or even years. During this phase, many newly produced capillaries relapse, restoring the wound to normal vascular density, and the essential component of the remodeling phase is ECM remodeling, which approaches normal tissue remodeling.

Phase IV: Remodeling

The remodeling phase is the final and longest stage of the wound healing process and it is predominantly determined by tissue breakdown and ECM formation. During the Cell proliferation slows down, protein synthesis drops and collagen is remodeled into larger molecules during this period. As a result, due to less ECM formation, the number of wound macrophages decreases in the final stage of normal wound healing. Both the size and volume of the wound will shrink as a result of these modifications and the wound's contraction.

WOUND PATHOLOGY

The integrity of biological tissue, such as the skin, mucous membranes, and organ tissues, is damaged by a wound. These can result from a variety of traumas, and it's important to make sure that wounds are cleaned and properly dressed to prevent the development of infection and additional harm. Due to poorly treated traumatic wounds are regarded to be dirty-infected wounds. Pathogenicity and virulence of the microorganism play a role in how an infected wound develops.

various wounds support various communities of bacteria since different wounds do not all have the same circumstances. Contamination, colonization, and infection are the three unique outcomes that might follow the acquisition of microbial species via wounds. As a result, various rats' wounds are treated with medication and antibiotics.

CRI ANIMAL MODEL DEVELOPMENT FOR DRUG SCREENING

CRIs are known to dramatically increase mortality and delayed wound healing. To study the effect of interventions against combined injuries, the animal model needs to be developed. In thisstudy, efforts have been made to develop CRI model in experimental rats (Sprague Dawley) by exposing the animal to different doses of ionizing radiation (^{60}Co-gamma rays: 5Gy and 6Gy) followed by creation of dermal wounds (excision wound) on the dorsal skin surface. Animals were then observed for changes in body weight, wound area, and wound score. Further the changes in hematopoietic system, and inflammation were determined in skin and blood tissues in animals exposed to CRI.

CHAPTER 3

AIM & OBJECTIVES

AIM & OBJECTIVES

AIM

Effect of escalated doses of gamma radiation in combined radiation and wound injury animal model.

OBJECTIVES – To study the effect of CRI injury by observing the following parameters:

1. **Physical parameters**

- Body weight
- Survival
- Wound area

2. **Hematological analysis**

- Blood cells count using hemocytometer – WBC, RBC, Platelets
- DLC Count – Granulocytes and Agranulocytes
- Blood culture analysis
- Bone marrow – Cell count, Apoptosis using flow cytometry

3. **Apoptotic damage in Bone Marrow using**

➢ Staining method
➢ Flow cytometry

4. **Histopathology**

- Technique learning and preparation of slides

➢ Tissue processing
➢ H&E Staining

5. **Biochemical assays in skin tissue-** Malondialdehyde (MDA), reduced glutathione (GSH)
6. **Inflammatory markers** – IL-1beta, IL-6 and IL-8
7. **Protein quantification and expression studies**

➢ BSA standard curve for protein quantification
➢ Western blotting – Technique learning and performing SDS PAGE
➢ Immunohistochemistry – Technique learning

CHAPTER 4

MATERIALS & METHODS

MATERIALS & METHODS

CHEMICALS REQUIRED

Milli Q water, Hematoxylin and Eosin alcoholic dyes, Xylene, Chloroform, Paraffin wax, Glacial acetic acid, 3% Hydrogen peroxide, 1M sodium hydroxide (NaOH), Absolute alcohol (100%), Bovine Serum Albumin (BSA), Methanol, DAB substrate, Distilled water, Glycerol

Tri-sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH=6.0), 1000 mL

Tri-sodium citrate (dihydrate), 10 mM	2.94 g
Distilled water	1000 mL

Mix to dissolve. Adjust pH to 6.0 with 1N HCl.

Add 0.5 ml of tween 20 and mix well.

10% neutral buffered formalin (NBF), 100 mL

37% formaldehyde solution	10ml
NaCl	0.8 g
Potassium phosphate monobasic	0.4 g
Potassium phosphate dibasic	0.65 g
Distilled water	90ml.

Phosphate Buffer Saline (PBS, ph:7.4), 1000 mL

NaCl	8 g
KCL	0.2 g
KH2PO4	0.24g
Na2HPO4	1.44g
Distilled water	1000 ml.

Leishman’s stain (Phosphate buffer, PH: 6.8), 1000 mL

Disodium hydrogen	20.214 gm
Sodium dihydrogen phosphate	3.394 gm
Distilled water	1000 ml.

EXPERIMENTAL ANIMAL- SPRAGUE-DAWLEY RAT

Figure8: Sprague-Dawley rat

(https://www.criver.com/sites/default/files/2017-11/CD_IGS_Rat417x235.jpg)

An outbred, multifunctional albino rat strain, the Sprague Dawley rat is widely utilized in medical research. Its tranquility and simplicity of use are its key advantages. The Madison, Wisconsin-based Sprague Dawley farms were the ones to develop this kind of rat originally. Gibco and Harlan both made separate purchases of the breeding facility in January 1980. Males weigh 450–520g and females between 250–300g as adults. The lifespan is typically 2.5 to 3.5 years.

- **Animal handling**

Animals were handled gently with gloved hands and a relaxed and calm body, holding them by the end of the tail. Laboratory mice and rats were handled on a frequent basis; this includes transporting them between their living quarters and testing locations, handling them during experimental procedures, and daily care activities including cage cleaning and supervision. Cages were kept in a humidity temperature control and aerated room with access bedding, sterilized food, filtered tap water, and good hygiene also rats were monitored for their body weight, infection, and physical activity.

COMBINED RADIATION INJURY MODEL

Animal grouping:

Rats were randomly divided into six groups, sham irradiated, only-excision wound, radiation alone (5Gy & 6Gy), radiation + excision (5Gy+EW & 6Gy+EW).

Radiation treatment:

The rats were first anesthetized using a ketamine-xylazine cocktail and then placed in a cobalt-60 radiation unit chamber and irradiated. To establish and develop a radiation combination injury model, the entire body of the animal was exposed to 5Gy & 6Gy in an LD2000 gamma radiator, and shortly after, a full-thickness excision wound was made.

Figure 9: LDI 2000 Co (60)-gamma radiator

Excision wound creation:

Following irradiation treatment, the hair from the dorsal surface was removed with a blade, and a circular excision wound of 2 cm diameter was created using a scissor. Diclofenac (an analgesic) injection was administered to rats after wound formation to reduce pain, and they were subsequently moved to their respective cages. The experimental rats were monitored daily for any changes in body weight, food, and water intake, or any indications of radiation illness.

Animal dissection

After observing the animals for 30 days, they were dissected to isolate organs such as skin, liver, kidney, small intestine, spleen, thymus, blood, and bone marrow.

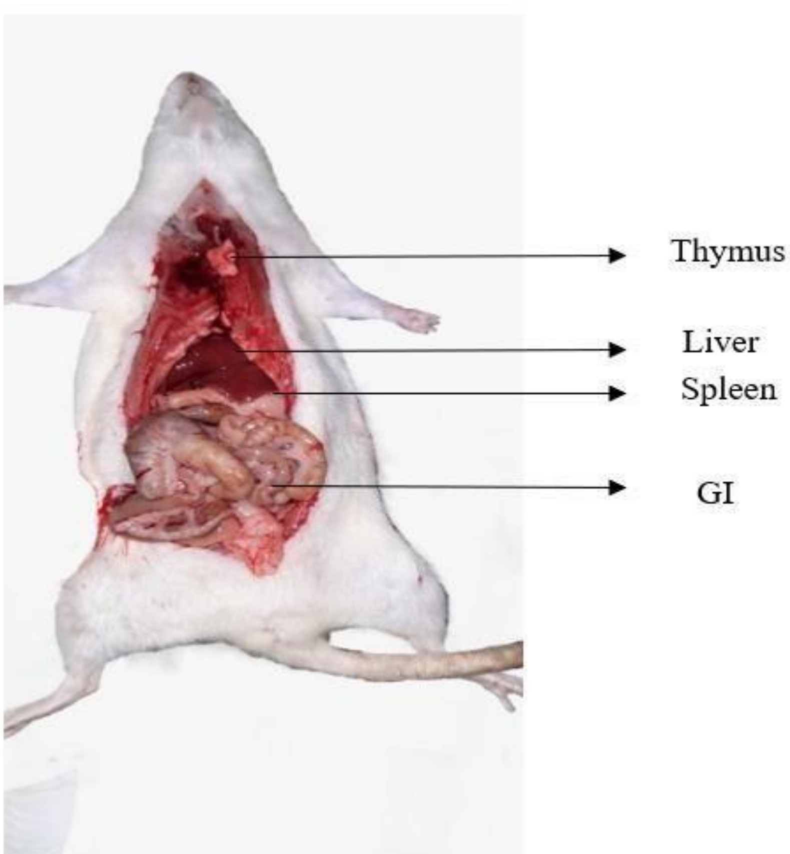


Figure 10: Dissected animal

PARAMETERS OBSERVED:

- **Wound area and body weight**

From day 0 until wound closure, the animals' survival rate, body weight, wound score, and wound area were observed. Body weight was recorded on trace paper together with the size of the wounds on days 0, 7, 15 and 30 following the radiation wound. Graph paper was used to measure the size of the wound. For the purpose of grading the wound, clinical indicators such as eschar formation, fluid discharge, bleeding, slough formation, hair growth, and odema were noted. The wound may appear dry or wet, and the presence of moisture in the wound may indicate how serious the injury is. The inflammatory phase of wound healing is indicated by swelling, odema, and slough appearance around the site. At this stage, the wound is extremely susceptible to infection, which is manifested by fluid discharge, black eschar, and pus formation.

- **Wound score:**

Table 1: Clinical scoring of the wound. (Adapted from Gupta and Tyagi, 2021).

Feature of wound bed	wound bed scores		
	0	1	2
Eschar formation	No	Little on edges	Thick
Odema/ swelling	No swelling	Slight swelling	Heavy swelling
Fluid discharge	No discharge	Little discharge	Heavy discharge
Appearance of pus/ slough	No	Slight presence	Heavy presence

Materials and chemicals required: Experimental animal, scissors, forceps, 5ml syringe, paper pins, dissecting foam board covered with a sheet, biohazard bag, scintillation vials, microcentrifuge tubes (1.5ml), petri dish, PBS (Phosphate buffered saline), 10% NBF (Neutral buffered formalin), chloroform, etc.

Reagents prepared

10% NBF (for 1000ml) (pH – 6.8)

37% Formaldehyde - 100ml

Sodium phosphate (monobasic) – 4gm

Sodium phosphate (dibasic) – 6.5gm

Distilled water – 900ml

PBS (for 1000ml) (pH – 7.4)

NaCl – 137mM

KCl – 2.7mM

KH_2PO_4 – 1.8mM

Na_2HPO_4 – 10mM

Procedure:

- Firstly, the experimental animal was sacrificed in CO_2 chamber.
- The rat was dissected on dissection board.
- The skin was picked up carefully in the middle of the ventral surface using forceps and was cut with the blunt end of the scissors.
- The desired organs such as the liver, kidney, and spleen, were carefully extracted and were put in PBS for washing and then stored in 10% NBF and at -80 degree refrigerator.

Instrumentation:

- Water bath
- Incubator
- Microtome
- Microscope
- Weighing balance
- Tissue flotation bath
- pH meter
- Vortex shaker
- Magnetic stirrer
- Centrifuge machine

HEMATOPOIETIC DAMAGE STUDIES

1. Hematology

1.1 Differential Leukocyte Count (DLC)

A White Blood Cell (WBC) differential leucocyte count (DLC) determines the proportion of each kind of WBC in the blood. Granulocytes, which include neutrophils, eosinophils, and basophils, and Agranulocytes, which include monocytes and lymphocytes, are the two different categories of white blood cells. We are able to assess any alterations caused by combined radiation injury to the immune system or blood cells using DLC.

Using DLC, we can examine any changes in the immune system or blood cells brought on by radiation injury together. Here, Leishman stain is being used. W.B. Leishman created the neutral stain known as the Leishman stain. It is often diluted and buffered throughout the staining process and comprises a mixture of eosin (an acidic stain) and methylene blue (a basic stain) in methylene blue. Leishman stain is frequently employed when it's necessary to check a blood smear for distinct blood cells, platelet count, etc., as well as to distinguish between the nuclear and cytoplasmic morphology of different blood cells like platelets, RBCs, and WBCs. Where the fundamental cell component, such as the cytoplasm, granules, etc., are inconsistently stained by the acidic dye eosin. Also, a differential leukocyte count can help to spot abnormalities in the distribution of white blood cells that could be indicative of particular diseases. Additionally, it provides information about the severity of the illness and the body's level of reaction.

Materials required: Blood sample, Leishman stain, methanol, DPX, tap water, clean glass slides, cover slips, Coplin jars, micropipette, and tip box.

Reagents prepared

Leishman stain:

- Weigh Leishman powder (0.15g) & add it to 100ml of acetone free methanol.
- Mix the solution for 10 minutes in mortar & pestle.
- Now filter the solution with the help of a funnel with filter paper rolled inside it with the help of a dropper.

- Now incubate at 50ºC for 15 minutes.

Procedure

1. Blood smear preparation:

- Blood was collected from the rat's heart after its dissection.
- 10ul of blood was taken on the slide and smeared with another glass slide by putting it at an angle of 45 degrees.
- Slides were fixed with the help of methanol for 5 minutes.

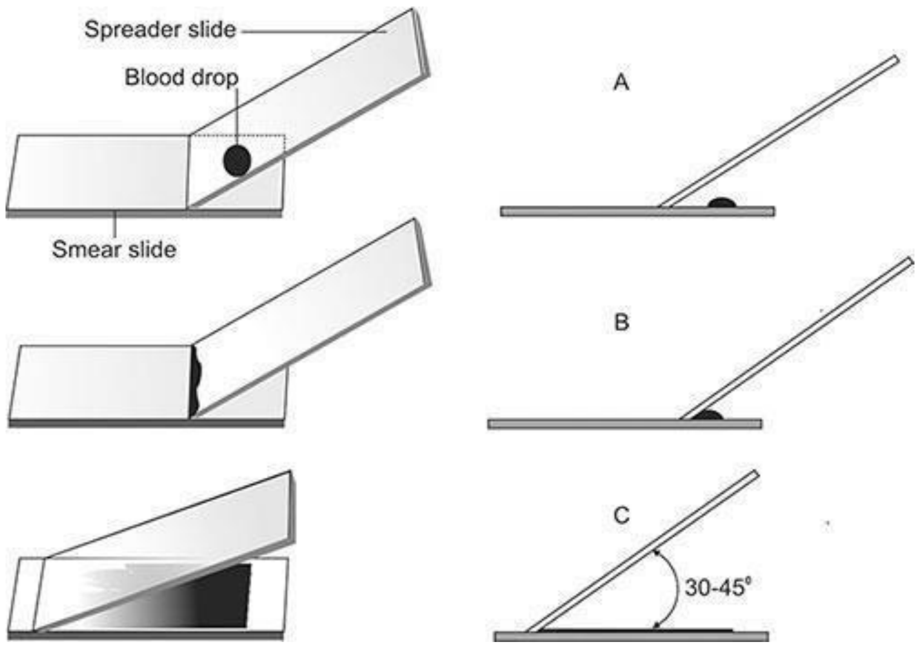


Figure 11: Preparation of blood smear

(https://d45jl3w9libvn.cloudfront.net/jaypee/static/books/9789380704470/Chapters/images/57-1.jpg)

2. Leishman staining:

- The undiluted Leishman stain was used for the staining purpose in respect to estimating the DLC.
- The blood-smeared slides were kept in a Leishman stain for 5-7 mins.
- The slides were washed with distilled water for 10 mins.
- Rinsed with tap water.

- After washing, the slides were blot dry.
- Mounted the slides with DPX and covered it with a cover slip.
- The slides were examined under the microscope.

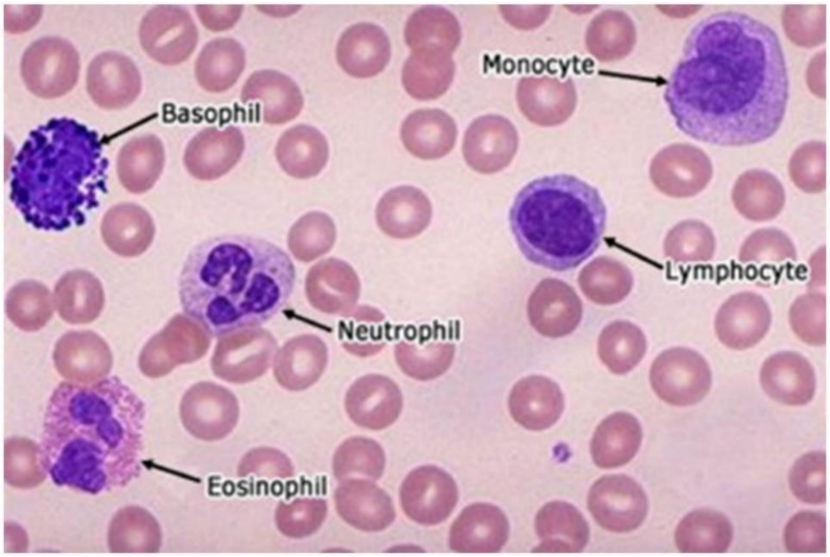


Figure 12: Different morphologies of leukocytes.
(https://slideplayer.com/slide/9718045/31/images/6/Blood+smear+under+microscope.jpg)

1.2 Blood cells count using a hemocytometer

A thick glass microscope slide with a grid of parallel lines etched in the middle serves as the basis of a hemocytometer. Because the grid has precise dimensions that clearly indicate how much area the lines enclose, it is straightforward to count the number of cells in a certain volume of the solution. There are nine primary squares on the hemocytometer, each measuring 1 mm by 1 mm. A red square designates the four corner squares, which are then divided into four 4*4 grids. Where red blood cells are often too few and tiny for this approach, thus they are placed in the middle square. Platelets use the middle square, whereas white blood cells use the 4 square.

A 1mm*1mm chamber has a volume of 0.3mm3 or 10-4ml because the cover glass-formed chamber has a 0.1mm height. Using a pipette, add 15 to 20 ml of suspension between the hemocytometer and cover the glass to count cells.

Materials Required: Hemocytometer, 70% ethanol, cover slip, Turk's fluid, blood sample, RBC diluting fluid, Platelet diluting fluid.

Reagents prepared:

a) Turk's fluid (for WBC)

   2% acetic acid – 100ml

   2-3 drops of gentian blue stain or methylene blue

b) RBC diluting fluid

   Trisodium citrate – 3.1g

   Formaldehyde – 1ml

   Make upto 100 with distilled water

c) Platelet diluting fluid

   Tri sodium citrate – 3.8g

   40% formaldehyde - 0.2ml

   Make upto 100 with distilled water

Procedure:

1. For WBC count

Hemocytometer was sterilized using 70% ethanol and was set under the microscope. 380µl of the Turks fluid was combined with 20µl of blood sample and mixed well. 10µl suspension was loaded between the hemocytometer and the cover slip using a pipette at the edge of the chamber. WBCs were counted in the four corners.

2. For RBC count

Hemocytometer was sterilized using 70% ethanol and was set under the microscope. 995µl of the RBC diluting fluid was combined with 5µl of blood sample and mixed well. 10µl suspension was loaded between the hemocytometer and the cover slip using a pipette at the edge of the chamber. RBCs were counted in the middle square.

3. For platelets count

Hemocytometer was sterilized using 70% ethanol and was set under the microscope. 380µl of the diluting fluid was combined with 20µl of blood sample and mixed well. 10µl suspension was loaded between the hemocytometer and the cover slip using a pipette at the edge of the chamber. Platelets were counted in the middle square.

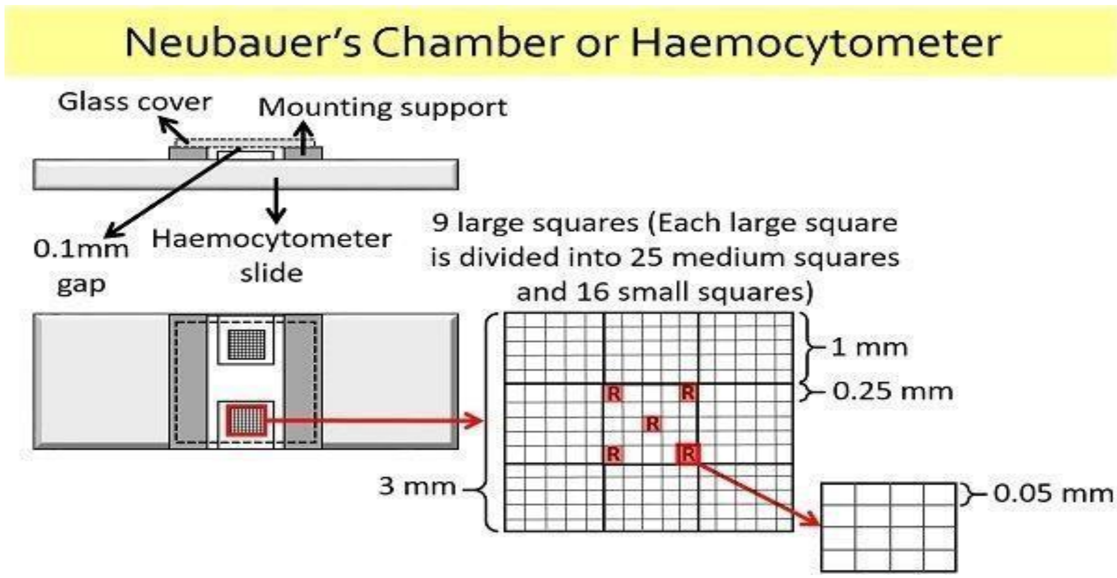


Figure 13: Hemocytometer

(https://biologyreader.com/wp-content/uploads/2021/03/Haemocytometer.jpg)

1.3 Blood culture analysis

Blood Agar (BA) is an enhanced media used to cultivate bacteria or other germs that are difficult to grow. These bacteria are referred to as "fastidious" because they require a different nutritional environment than typical bacteria. A wide variety of pathogens are grown on Blood Agar, especially those that are more challenging to culture, like Streptococcus pneumoniae, and Haemophilus influenza. The blood also helps to see various bacteria's hemolytic responses. On blood agar, four distinct types of hemolysis are seen, that is alpha hemolysis, beta hemolysis, gamma hemolysis, and wide zone alpha hemolysis, each of which can be distinguished by the presence of a hemolysis zone around the expanding colonies. The type of animal blood utilized, however, affects the hemolytic effects. In order to observe colony morphology and count the organism, agar is the hardening agent that creates a stable surface for the organism to develop on.

Materials required: Nutrient agar medium, Petri plates, Sterile tips, Micropipette, Autoclave Eppendorf, Ethanol, Laminar air flow, and Cell spreader.

Procedure

- Blood agar base powder (16 g) was measured in 300 ml of distilled water in 500 ml flask.
- Heat the mixture on a hot plate to fully dissolved all components.
- Autoclave the dissolved mixture at 121 degree celsius for 20 minutes.
- The media is then poured into the sterile plates as soon as possible, without air bubbles under sterile conditions.
- Now once the media was solidified, the plates were put into the incubator at 37 degree celsius temperature for overnight.
- 2ml of di-potassium EDTA was filtered using a 0.22-micron syringe filter in an eppendorf tube and 10µl filtered 0.5M EDTA was taken in each eppendorf tube to collect blood.
- Blood samples taken from all the experimental groups were taken on day 0, 7, 15, and 30 and were prepared for serial dilution in 0.9% saline.
- 100µl diluted blood sample was spread on solidified plates and allowed to dry in the laminar airflow cabinet for 5min.
- Plates were incubated overnight and after 24 hours, the colonies were counted.

Calculation:

(Colonies forming unit/ml= Number of colonies counted * dilution factor/ Volume plated.)

<u>BONE MARROW DAMAGE ANALYSIS</u>

1. May Grunwald-Giemsa staining

May Grunwald-Giemsa staining for bone marrow is the outcome of combining the May Grunwald and Giemsa stains. It is a type of Romanowsky stain and is used to study the cellular morphology, cytoplasm, granules, and basement membrane. The key ingredients in the alcohol-based May Grunwald stain are methylene blue and eosin. Methylene blue, eosin, and azure B are the main components of the alcohol-based stain known as giemsa. The cation (positively charged, basic) and anion (negatively charged, acidic) components of the MGG stain are methylene blue and the reagent-related thiazine dyes, such as azure B, in various ratios, respectively. Cells are initially fixed by the solvent methanol. The granules of basophil granulocytes, nuclei (due to the negative charges of phosphate groups of DNA and RNA molecules), and cytoplasmic RNA molecules are stained by the basic dyes because they have net positive charges. Red blood cells and the granules of eosinophil granulocytes are stained by the eosin, which has a net negative charge. In order to get the dye to precipitate and bind well with the cellular material, a buffer solution with a pH range of 6.5–6.8 is utilized.

Materials required: May Grunwald Stain, magnetic stirrer, giemsa stain, phosphate buffer saline, fetal bovine serum, and Slides.

Reagents preparation:

a) May Grunwald Stain

- 0.3g of May Grunwald dye in 100ml absolute methanol.
- Warm at 50ºC in a water bath & allow it to cool at room temperature.
- Stir the mixture on a magnetic stirrer & leave it stirring for 24 hours.
- Filter the mixture and it is ready for use.

b) Giemsa stain

- 1g Giemsa dye to 66ml of glycerol and warm the mixture for 1-2 hours at 50ºC.
- Cool the mixture at room temperature and add 66ml of methanol.
- Let the mixture to dissolve.
- Stain is ready to use.

Procedure:

- 1ml chilled phosphate buffer saline was used to aspirate bone marrow from the femur bone and centrifuge at 15000 rpm for 7 minutes.
- To suspend the pellet in fetal bovine serum, dilute it with 50µl to 100µl. For 15to 20 minutes, fix the bone marrow smear in absolute methanol.
- Eq. volume of may Grunwald and phosphate buffer (pH 6.8) poured onto the slide and stained for 10 minutes.
- 1 to 10 times dilution of giemsa stain with phosphate buffer was prepared. Mix well.
- Giemsa was poured on a smear for 15 minutes.
- After 15 minutes pour off the stain and flush slides with tap water.
- Observe the slides under a microscope.

2. Bone marrow dual fluorescent staining

In this study, AO/EB staining was evaluated to determine its effectiveness in detecting tumor cell apoptosis. It is clear that normal cells, early and late apoptotic cells, and necrotic cells are differentiated according to the changes in cell membranes associated with apoptosis. Acridine orange/ethidium bromide dual fluorescence staining is regarded as a quick, affordable, and simple technique to examine the viability of cells. Because of the strong negative charge of the phosphate groups, sugars, and hydrogen bonding possibilities in DNA and RNA, ethidium bromide's (EtBr) relative selectivity for nucleic acids is likely attributable to these factors, which are also used to explain acridine orange's (AO) high specificity for nucleic acids. Thus, the preparations using AO as fluorescent DNA-specific staining to nuclear DNA and micronuclei with green colour in tandem with carrying out viability testing are provided. A less time-consuming and at least similarly accurate observation was achieved with AO staining.

Materials Required: Phosphate buffer saline, Femur bone, Centrifuge, Fetal bovine serum, Methanol, Acridine orange, Ethidium bromide, Slides, Pipettes, and Tips.

Procedure:

- 1ml chilled phosphate buffer saline was used to aspirate bone marrow from the femur bone and centrifuge at 15000 rpm for 7 minutes.
- To suspend the pellet in fetal bovine serum, dilute it with 50µl to 100µl. For 15 to 20 minutes, fix the bone marrow smear in absolute methanol.
- For 2-5 minutes, the slides were stained with a dual mixture of 1ml 5mg/ml acridine orange and 1ml 5mg/ml ethidium bromide in 48 ml phosphate buffer saline.
- Slides were rinsed with phosphate buffer saline twice and examined with the help ofa microscope under a green and yellow filter.

3. Flow cytometry

Flow cytometry is a powerful and flexible technique that can rapidly analyze multiple parameters of individual cells, within heterogeneous cell populations. Flow cytometers are utilized in a range of applications, from immuno-phenotyping, ploidy analysis, and cell counting, to fluorescence-activated cell sorting (FACs), fluorescence expression analysis, and more. The flow cytometer performs this analysis by passing thousands of cells per second through a laser beam and capturing the fluorescence and scattered light that emerges from each cell. As the cells pass through, fluorescence data is collected and analyzed statistically by flow cytometry software to report cellular characteristics including size, complexity, phenotype and health (e.g., viability, proliferation and apoptotic states). Fluorescence is an important feature of fluorophores as it is used to differentiate their color, that is, the wavelength at which they emit fluorescent light. The ability to simultaneously utilize multiple fluorophore-conjugates with distinct and well-separated emission profiles permits multi-parametric analysis, which is the real power of flow cytometry.

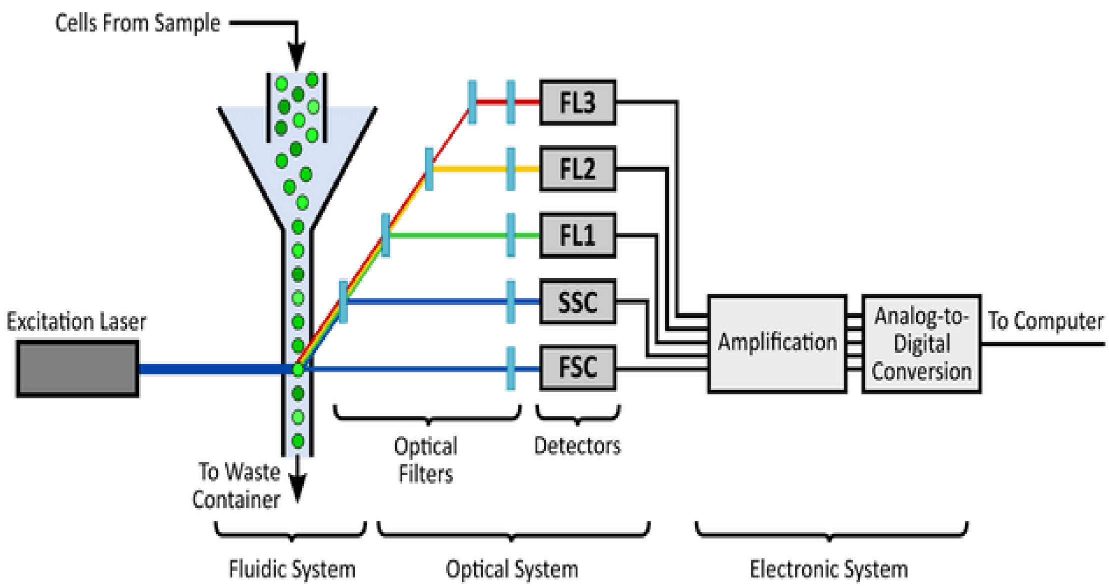


Figure 14: Schematic overview of common flow cytometry (https://microbenotes.com/wp-content/uploads/2020/05/Flow-Cytometry-Definition-Principle-Parts-Steps-Types-Uses.jpg)

Materials Required: Vybrant apoptosis kit, Femur bone, PBS, Flow cytometry tube wells, 1X annexin binding buffer, Propidium iodide, Pipettes, and Tips.

Procedure

- Apoptotic cells detection was performed by the commercially available Vybrant apoptosis assay kit as per the instructions of manufacturers. Femur bone cells were resuspended in the phosphate buffer saline.
- Add 200 µl of annexin binding buffer in the tube wells, 10 µl of annexin V conjugate, and 2.5 µl propidium iodide to each tube wells.

- Incubate at room temperature for 15 minutes.
- Wash cells with 1X annexin binding buffer and samples were analyzed in the flow cytometry.

HISTOPATHOLOGICAL ANALYSIS

Histopathology is the study of a biopsy or surgical material by a pathologist following the processing and placement of histological sections onto glass slides. It is a branch of clinical medicine. It is the study of disease symptoms through histopathology and involves a microscopic inspection of a surgical or biopsy specimen that has been processed and put onto glass slides. In this, the tissue must first be processed and fixed in a wax cube before being sliced into slices and stained with H&E for microscopic examination.

Materials Required: Tissue, Phosphate buffer saline, 10%NBF, Knife, 70% ethanol, 95% ethanol, Absolute ethanol, Xylene, Paraffin wax, Tissue cassette, Incubator, Microtomy, Water bath, Glass slide, Mayer's albumin, Gill's Hematoxylin, DPX mounting medium.

Procedure:

Step1. Fixation of tissue

Organs of interest were isolated from the rats during dissection and were immediately flushed with ice-cold PBS to remove any digest material and were stored in 10% NBF for 24 hours (max 36 hours) at room temperature for fixation. (NOTE: fixative volume should be 20 times that of tissue on a weight-per-volume scale)

1. The tissues were cut with a microtome blade/ knife or fresh surgical blade in 0.5cm sections and washed 3 times with 70% ethanol.
2. Dehydration process:

- 75% ethanol: 2 changes of 30 minutes each
- 95% ethanol: 2 changes of 30 minutes each
- 100% ethanol: 2 changes of 30 minutes each
- xylene: 2 changes of 30 minutes each

  (Note: xylene incubation is very critical. Xylene is a clearing agent. Longer time incubation in xylene can make tissue very brittle. Sometimes 2 changes of 20 minutes are also sufficient).
- Xylene + paraffin wax: Incubation for 2 hours

(Note: Ratio of xylene/ paraffin wax= 1:1 Gentle agitation improves the penetration of the ‘xylene + paraffin wax’ mixture in tissue)

- Liquid paraffin wax: Incubate overnight/ 16-18 hours at 60ºC.

(Note: use paraffin wax with melting temperature 56-58ºC. For incubation in liquid paraffin wax, keep the tissue in an incubator set at a temperature of 62-64ºC. That’s how the temperature of liquid paraffin wax will reach 60º. Gentle agitation improves the penetration of paraffin wax in tissue).

Step 2. The casting of tissue wax blocks

- Cassette was opened to view the tissue samples and choose a mold that best corresponds to the size of the tissue. A margin of at least 2mm of paraffin surrounding all sides of the tissue gives the best cutting support. Discard cassette lid.
- Molten paraffin was put in the mold. Using warm forceps, mold was transferred into the cold plate, and gently press the tissue flat. Solidified paraffin in a thin layer that holds the tissue in position.
- When the tissue was in the desired orientation, add the labeled tissue cassette on the top of the mold as baking. Press firmly. Add hot paraffin wax to the mold.
- Paraffin solidified in 30 minutes. When the wax is completely cooled and hardened (30 minutes), pop the paraffin wax blocks out of the mold. (If the wax cracks or the tissues are not aligned well, simply melt them again and start over).

Step 3. Microtomy

- Water bath was turned on and set the temperature to 50ºC. Use fresh deionized water (DEPC-treated water must be used if in situ hybridization will be performed on the sections).
- Wax blocks were placed face downwards on the melting ice cube for two minutes.
- The blocks inserted into the microtome chuck so the wax blocks face the blade and was aligned in the vertical plane.
- Blocks were trimmed and cut 10µm in tissue sections. Once it was cut smoothly a fresh blade placed on the microtome; set it to 5µm and cut tissue sections. (The blade should be angled at 5ºC to 15ºC).
- Blocks were ribbon then cut another four sections and pick them up with forceps or a fine

paint brush, float them on the surface of the 50ºC water bath.

- Float the sections onto the surface of clean glass slides [pre-coated with Mayer's albumin (50% egg albumin + 50% glycerol) for H&E staining or with gelatin for other applications].
- Blocks were not ribbon well then placed it back on the melting ice cube for 10-15 sec and cut the sections.
- The slides were placed with paraffin sections at 65ºC incubator/oven for 20 minutes (so the wax just starts to melt) to bond the tissue to the glass slide (or keep the slides with paraffin sections at room temperature overnight).
- Slides was stored overnight at room temperature.

Step 4. Hematoxylin & Eosin (H&E) staining

- Xylene : 3 changes of 5 minutes each
- 100% ethanol : 2 changes of 5 minutes each
- 95% ethanol : 2 changes of 1 minute each
- 75% ethanol : 1 minute
- 50% ethanol : 1 minute
- Milli-Q water : 10 minutes (minimum)
- Gill's Hematoxylin 2.0 : 30 minutes
- Tap water : 60 minutes
- Eosin alcoholic : 10 to 30 dips
- 75% ethanol : 30 dips
- 100% ethanol : 3 changes of 1 minute each
- xylene : 3 changes of 1 minute each
- Mount in DPX mounting medium and cover slip.

WOUND HEALING ASSESSMENT IN SKIN

1. Inflammation studies

1.1 MPO assay

It is a quantitative assay for determining the activity of myeloperoxidase in a sample. Myeloperoxidase (MPO) is a heme-based peroxidase enzyme with an antibacterial action against a wide spectrum of species. MPO is abundantly expressed in activated neutrophil granulocytes, where it catalyzes the formation of hypohalous acids such as hypochlorous acid (HOCl) from hydrogen peroxide (H2O2) and chloride ion (Cl) or other halides, then it quickly interacts with taurine to form a stable taurine chloramine product. Finally, taurine chloramine reacts with the yellow TNB chromogen probe, with a reduction in colour suggesting increased MPO activity.

Materials Required: Tissue, Potassium phosphate buffer, Centrifuge, Sodium phosphate buffer, 96 well plates.

Procedure:

- Wound tissue sample of the animal was weighed 0.02g and homogenized with 10 vol. ofice-cold potassium phosphate buffer (50mM) at (PH 6) containing CTAB (0.5%w/v).
- Centrifuge at 5000g for 10 minutes at 4°C, then discard the supernatant. Resuspend the supernatant in 500µl of 50mM potassium phosphate buffer containing (0.5% CTABand 10mM EDTA) was added to the pellet.
- Freeze and thaw up to three times, then sonicate for 15seconds. Centrifuge for 15 minutes at 4 degrees Celsius at 15000g.
- Add 290µl of sodium phosphate buffer (0.2M) containing O-dianisidine dihydrochlorideand 0.0005% H2O2 to 10µl supernatant extract sample.
- Enzyme activity was estimated by measuring the absorbance at 460nm. MPO activity data are presented as units per gram of weight tissue.

Calculation:

MPO activity (u/g) = X / weight of tissue

X = (10× Change in absorbance /min.) / (Volume of supernatant taken in final reaction)

2. Quantitative estimation of IL6, IL8, IL-1beta using Sandwich ELISA

This assay was performed by using BT Lab sciences ready-to-use kit. The protocol was followed as per the manufactures instructions. ELISA is extremely effective in detecting sample antigens. It measures antigens that are sandwiched between two layers of antibodies. The two antibodies act in the sandwich form, so the antigen to be assessed must include at least two antigenic epitopes capable of binding to antibodies.

Materials Required: Eppendorf tube, 96 well plates, Standard solution, Diluent solution, Streptavidin HRP, Sample, Substrate A, Substrate B, Stop solution, and BT Lab sciences kit.

Procedure:

- A standard curve was plotted for IL-6, IL-8, and IL-1betaby using BT Lab sciences ready to use kit as per given manufacturer instruction. 120 µl of the standard was diluted with given diluents, and further were two folds serial diluted.

Table 2: Table representing standard and diluent volume used for IL6 standard curve.

CONCENTRATION (ng/l)	**SSTANDARD VOLUME (µl)**	**DILUENT VOLUME (µl)**
48ng/l	120 µl	120 µl
24ng/l	120 µl	120 µl
12ng/l	120 µl	120 µl
6ng/l	120 µl	120 µl
3ng/l	120 µl	120 µl
0ng/l	0 µl	120 µl

- 50µl of different standard concentrations were taken in a standard well and 50µl of streptavidin-HRP was added and incubated for 60min at 37 degree celsius.
- Washing was done after 60 minutes 4-5 times and 50µl substrate A and B was added and the wells were incubated for 10 minutes and to end the reaction.

- 50 µl stop solution was added. Within 10 minutes. OD was taken at 450 nm. A standard graph was plotted between concentration and absorbance, and the slope and R square values were calculated.

3. Oxidative stress analysis

3.1 MDA (Malondialdehyde)

This assay was performed by using Bio Vision ready-to-use kit. MDA is a lipid peroxidation marker that is used to detect lipid peroxidation caused by increased oxidative stress. Malondialdehyde (MDA) is a byproduct of polyunsaturated fatty acid peroxidation in cells. The thiobarbituric acid (TBA) assay is the most extensively used method for determining MDA in biological fluids. The assay is based on a condensation reaction between two molecules of TBA and one molecule of MDA, the rate of which is affected by temperature, pH, and TBA concentration. Due to overproduction of MDA is caused by an increase in free radicals. Malondialdehyde levels are often used in cancer patients as a measure of oxidative stress and antioxidant status.

Materials Required: TBA, deionized water, Eppendorf tube, 96-well plate, Sample, Incubator, and Bio Vision kit.

Reagent Prepared:

- 0.37% TBA- 0.37gm/100ml
- Phosphate Buffer Saline (80gm Nacl, 11.6gm Na_2HPO_4, 2gm KH_2P0_4 and 2gm KCL) in 10-liter deionized water at PH-7.

Procedure:

- Diluted 20 µl standard solution with 980 µl deionized water in an eppendorf tube wasmixed to make a working solution (2mM) by mixing 10 µl stock solution with 407 µl water.
- A standard curve was plotted by using Bio Vision ready-to-use as per given manufacturer instructions. Add 200µl solution in each 96-well plate and calculate at 532nm using 600µlTBA diluted and 200µl deionized water diluted from (2mM) working solution.

Table 3: Table representing standard working solution and thiobarbituric acid volume used.

S.No.	WORKING SOLUTION (mM)	DEIONIZED WATER (µl)	THIOBARBITURIC ACID (TBA) (µl)
1	0Mm	200 µl	600 µl
2	0.02mM	200 µl	600 µl
3	0.04mM	200 µl	600 µl
4	0.06mM	200 µl	600 µl
5	0.08mM	200 µl	600 µl
6	0.1 mM	200µl	600µl

- Diluted 200µl deionized water with 20µl sample and (2mM) working solution in 96-well plates. Fill each well with 600µl TBA solution.
- Incubate at 95˚c for 60 minutes and cool at room temperature. Take OD at 532 nm.

Table 4: Table representing working solution and thiobarbituric acid volume used.

S.No.	WORKING SOLUTION (mM)	DEIONIZED WATER (µl)	SAMPLE VOLUME (µl)	THIOBARBITURIC ACID (TBA) (µl)
1	0mM	200 µl	50 µl	600 µl
2	0.02mM	200 µl	50 µl	600 µl
3	0.04mM	200 µl	50 µl	600 µl
4	0.06mM	200 µl	50 µl	600 µl
5	0.08mM	200 µl	50 µl	600 µl
6	0.2 mM	200µl	50 µl	600µl

Calculation

The concentration of MDA (nmol/mL) in sample = [Sa/Sv ×4 ×DF]

where: Sa = Amount of MDA in Sample (nmole) as determined from the standard curve Sv= Sample volume (mL) or amount (mg) added into the wells 4 = Correction factor for using 200 µL of the 800 µL reaction DF = Sample dilution factor (DF = 1 for undiluted Samples).

3.2Glutathione (GSH)

An indication of oxidative stress is the intracellular quantity of GSH, a crucial tripeptide thiol antioxidant. It is essential for the transmission of signals, gene expression, and apoptosis. This test is based on DTNB's (also known as Ellman's reagent) reaction with GSH to create TNB chromophore and oxidized glutathione-TNB. The disulfide product (GS-TNB) is subsequently reduced by GR in the presence of NADPH, recycling GSH back into the process since GR reduces the GSSG produced into 2 GSH. As a result, the glutathione concentration measured in the sample represents the total quantity of reduced and oxidized glutathione.

$$([GSH]total = [GSH] + 2\times[GSSG])$$

Materials Required: 96-well plate, Homogenate sample, Orthophosphate solution, DTNB solution, Pipettes, Tips.

Reagent Prepared:

- Orthophosphate solution- 0.3M Na_2HPO_4 in 100ml deionized water.
- Precipitating reagent- 1.67g metaphosphoric acid, 0.2g EDTA, 30g Nacl in 100ml deionized water.
- DTNB solution- 40mg in 0.5% of 100 ml sodium citrate.

Procedure

- 20 µl standard GSH or tissue homogenate was taken and to that 180 µl deionized water and 300 µl ppreagent were added and it was allowed to stand for 5 min. at room temperature.
- Centrifuge at 4000 rpm for 15 min.

- 200 µl of filterate was taken. To that 800 µl phosphate solution and 100 µl DTNB solution were added. OD was taken at 412 nm.

PROTEIN QUANTIFICATION

Bradford-protein quantification assay

A Bradford test detects proteins by using the Coomassie Brilliant Blue G-250 dye. The dye is present in three different forms: cationic (red), neutral (green), and anionic (blue). It is primarily in the doubly protonated red cationic form (Amax = 470 nm) under acidic circumstances. When the dye binds to the protein, it is transformed into a stable unprotonated blue form (Amax = 595 nm) that may be measured with a spectrophotometer or microplate reader. Therefore, the spectrophotometer or microplate reader detects the blue protein-dye at 595 nm with the help of Beer's law to determine protein concentration by selecting an appropriate dye volume to concentration ratio.

$$\text{(Beer Lambert law) } A = \varepsilon c L$$

Calculations:

(Net Absorbance = Absorbance value of the protein sample — Absorbance value of blank).

Materials Required: 96- wells plate, BSA stock solution, deionized water, Sample, Bradford reagent.

Procedure:

- Plot a standard curve at 595nm, add 200µl bradford reagents in 96- wells plate, and diluted from 1mg/ml BSA stock solution with 20µl deionized water. The color changes from brown to blue. The volume of BSA for the standard equation has been added as per the table

Table 5: Table depicting the concentration of BSA and bradford used.

S.No.	BSA STOCK SOLUTION (mg/ml)	VOLUME OF BSA (µl)	BRADFORD REAGENT (µl)
1	1mg/ml	20µl	200 µl
2	0.5mg/ml	20µl	200 µl
3	0.25mg/ml	20µl	200 µl
4	0.125mg/ml	20µl	200 µl
5	0.0625mg/ml	20µl	200 µl
6	0.0314mg/ml	20µl	200 µl
7	0mg/ml	20 µl	200 µl

- Afterwards, dilute 2µl of sample with 18µl of water and add 200µl of Bradford reagent then wait for 5 min. before loading into a 96-wells plate and calculating OD at 595 nm. The color changes from brown to blue.

The concentration of the protein sample corresponding to the absorbance obtained was calculated using the standard equation and the net absorbance values obtained for the protein sample. This was multiplied by a dilution factor to estimate the protein in actual the amount of sample, which gives the concentration of protein from BSA main stock, was made, and a standard curve.

WESTERN BLOTTING

Protein immunoblotting is another name for Western blotting, which uses an antibody to identify an antigen. It is a frequently used analytical method for identifying particular proteins in a sample. DS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate the different proteins in the provided sample. The separated proteins in the gel are electrophoretically transferred to a membrane that has been put on the gel following SDS-PAGE. Following a wash and a reaction with a secondary antibody labelled with an enzyme, such as horseradish peroxidase (HRP), the membrane containing the transferred proteins is probed with a primary antibody. A chemiluminescent or chromogenic technique is utilized to identify the target protein using the bound enzyme activity.

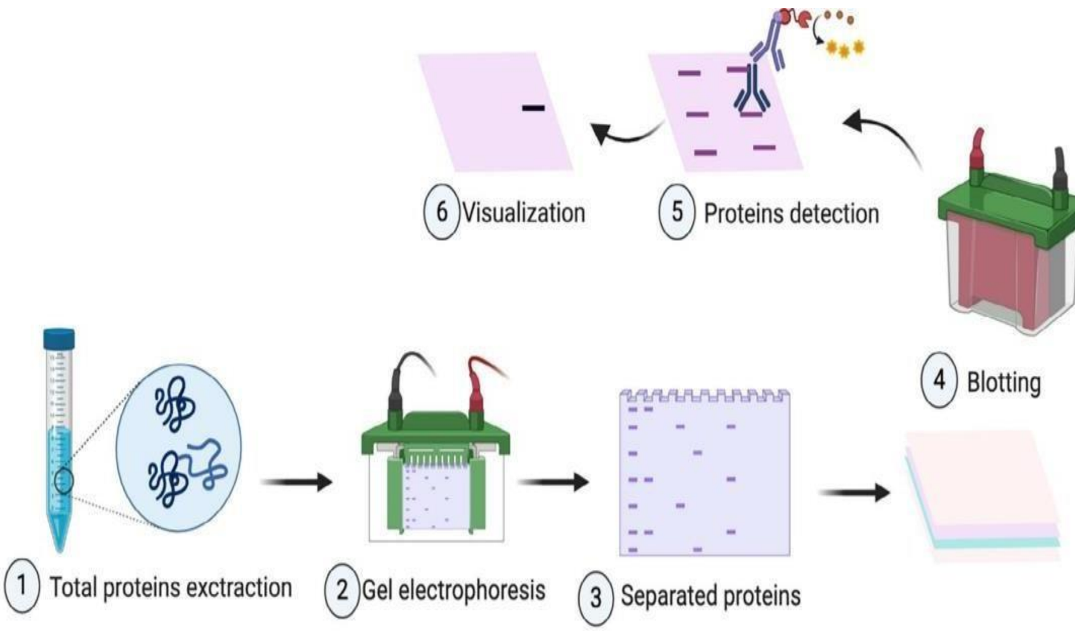


Figure 15: The workflow of the western blot technique. (https://iubmb.onlinelibrary.wiley.com/cms/asset/2c69c13e-613e-47a5-bc1e-af4db0ce5f3a/bmb21516-fig-0001-m.jpg)

Materials Required: Lysis buffer, sample, Loading buffer, Blotting paper, Sponge, PVDF (Polyvinylidene Fluoride) membrane, Running buffer, Transfer buffer, Primary antibody, Secondary antibody, Stacking gel, Separating gel, Coomassie brilliant blue R-250, Blocking buffer, Mini protean apparatus, Shorter plates, and Comb.

Reagent Prepared:

- **Lysis buffer**

To prepare lysis buffer, we utilised100ml of RIPA buffer consisting of:

50ml of 100mM Tris Hcl (PH-8) protease inhibitor, 0.876 g of 150mM NaCl, 1% Triton X-100 0.5g of Sodium deoxycholate, 0.1g of SDS.

- **Lamelli 2X buffer/ Loading buffer**

Prepare for 100ml loading buffer- 4g SDS, 10g of 2-mercaptoethanol, 20g glycerol, 0.04g bromophenol blue, 65 ml of 200mM Tris Hcl at (PH-6.8).

- **Running buffer**

Prepare 100ml of running buffer- 0.303g Tris base, 1.426g glycine, and 0.1g SDS.

- **Separating gel prepared**

Prepare 10 ml resolving gel- 3.2ml Deionized water, 2.6ml of 1.5M Tris Hcl (PH 8.8), 2ml of 40% acrylamide, 200µl of 20%SDS, 20µl of 30%APS and 8µl of TEMED.

- **Stacking gel prepared**

3.9ml of Deionized water, 500µl of 1M Tris Hcl at (PH-6.8), 500µl of 40% acrylamide, 100µl of 20% SDS, 16µl of 30%APS and 8µl TEMED.

- **Staining- Destaining solution**

Prepare Coomassie stain solution: 150ml Ethanol, 50ml Glacial acetic acid, 300ml Deionized water, 1g Coomassie brilliant blue R-250.

Prepare destaining solution- 120ml Ethanol, 400ml Glacial acetic acid, and 2.4 liter Deionized water.

- **Transfer buffer**

Prepare 100 ml transfer buffer (wet)- 0.303g tris base, 1.426g glycine, and 20ml methanol.

- **Blocking buffer**

Prepare 100ml TBS solution- 2.4g Tris Hcl, 5.6g Tris base, 8.8g Nacl, 90ml water at (PH- 7.6). Prepare 100ml TBST solution- 10ml TBS 10X, 90ml Deionized water, and 1ml Tween 20 Prepare 3-5% BSA and add to TBST buffer then mix and filter the solution.

Procedure:

1. **Preparation of lysate from tissues:** 5mg of tissue was dissected and weighed; and to it 300µlof ice-cold commercially available RIPA lysis (Sigma-Aldrich) was added and homogenized.
2. **Protein quantification:** 1mg/ml BSA stock was prepared and a standard curve was plotted by serial diluting the stock concentration of BSA. For sample quantification, samples were diluted with deionized water and 10 times the volume of the sample, Bradford reagent (biorad) was added. After 5 minutes, OD was taken at 595 nm.

3. **Protein separation:** equal volume of 2x lamelli buffer was added to the sample and cell lysate was boiled at 100 degree for 5 minutes.

SDS PAGE

- Glass plates and spacers were assembled and the resolving gel mixture was poured into gel plates to a level 2cm below the top of the shorter plate.
- A layer of distilled water was placed over resolving gel and then the gel was allowed to stand for 30 minutes at room temperature.
- Distilled water was drained from the top and stacking gel was poured on top of it. The comb was inserted and the stacking gel was allowed to stand.
- The cast gel was assembled into mini protean apparatus (Biorad) and freshly prepared running buffer was added to the chamber.
- Denatured Samples were loaded into the wells and the gel was allowed to run for 30 minutes at 70V and 90 minutes at 80V or till the time the dye reaches the bottom of the gel.

4. **Coomassie staining and destaining:** 20 ml of staining solution was poured onto the gel for 40-50 minutes with gentle shaking. After 40 minutes, the staining solution was poured off and the gel was kept in the destaining solution for 2hrs.

5. **Transferring the protein from the gel onto the membrane:**

- The gel is taken out of the electrophoretic device and placed to a membrane made of polyvinylidene fluoride (PVDF)that can bind proteins.
- After calibrating the gel in the transfer buffer for 15 minutes, the PVDF membrane (Polyvinylidene Fluoride) was activated for one minute with methanol, then rinsed repeatedly with the transfer buffer before being applied to the membrane.
- The PVDF (Polyvinylidene Fluoride) membrane was sandwiched between the gel and the cassette of filter paper.
- Through capillary action that is aided by the flow of the electric current, the proteins in the gel are transported to the PVDF (Polyvinylidene Fluoride) membrane.

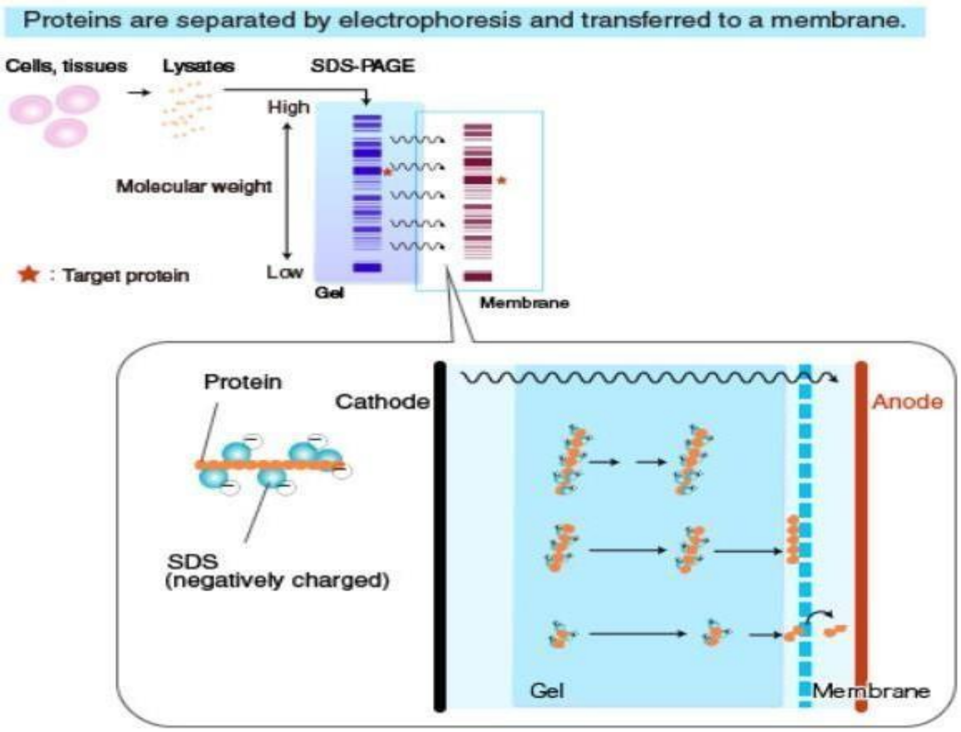


Figure 16: Transfer the protein bands to the PVDF membrane.

(**https://www.mblintl.com/wp-content/uploads/Principle45.png**)

6. Blocking and antibody staining

- PVDF (Polyvinylidene Fluoride) membrane was blocked with 5% BSA in TBS overnight at 4˚C after that incubated with the primary antibody (1:1000 dilution) in a blocking buffer.
- Washed three times with TBST for five minutes each the next day, with the aid of this radiolabeled antibody forms antigen-antibody complex with the desired protein.

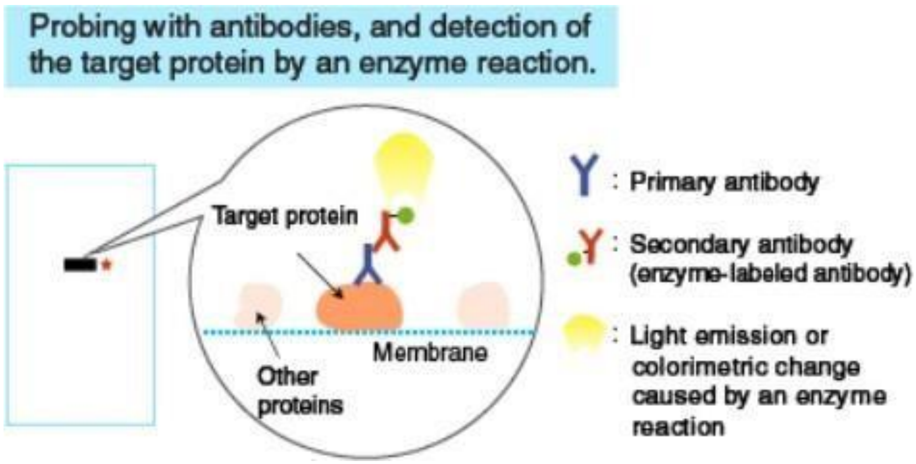


Figure 17: Blocking and probing with antibodies.

(https://www.mblintl.com/wp-content/uploads/Principle45.png)

7. Visualization of protein

- Incubate PVDF (Polyvinylidene Fluoride) membrane to conjugate secondary antibody for one hour at room temperature after wash 3 times with TBST solution for 5 minutes each.
- Afterward, again PVDF (Polyvinylidene Fluoride) membrane washed 3 times with TBST solution for 5 minutes each, and visualize the target protein using HRP (Horseradish Peroxidase) substrate with chemiDoc.

LEARNING TECHNIQUE

Immunohistochemistry

Immunohistochemistry (IHC) uses antibodies to detect the location of proteins and other antigens in tissue sections. The antibody-antigen interaction is visualized using either chromogenic detection with a colored enzyme-substrate, or fluorescent detection with a fluorescent dye. Although less quantitative than assays, such as western blotting or ELISA, IHC gives invaluable information about protein localization in the context of intact tissue. Protein expression patterns are tremendously valuable for pathologists and as diagnostic tools. Essential to a successful IHC experiment is a robust, optimized, and reproducible staining regimen that makes use of high-quality, specific reagents. We've put together this comprehensive guide to help you smoothly progress through all IHC steps, from tissue processing and blocking to detection and counterstaining. The guide also covers various troubleshooting scenarios so you can easily resolve any common IHC problems.

Materials Required:

Procedure

Step 1. Fixation of tissue

- Skin was isolated from the Sprague Dawley rats at the dorsal site during dissection and skin was stored in 10% NBF for 24 hours (max 36 hours) at room temperature for fixation.
- Tissue was cut with a microtome blade/ knife or fresh surgical blade in 0.5cm sections.
- Tissue was washed 3 times with 70% ethanol.
- Tissue was kept overnight in 70% ethanol.

Step 2. Dehydration process

- 75% ethanol: 2 changes of 30 minutes each.
- 95% ethanol: 2 changes of 30 minutes each.
- 100% ethanol: 2 changes of 30 minutes each.
- xylene: 2 changes of 30 minutes each.

(Note: xylene incubation is very critical. Xylene is a clearing agent. Longer time incubation in xylene can make tissue very brittle. Sometimes 2 changes of 20 minutes are also sufficient).

- Xylene + paraffin wax: Incubation for 2 hours

(Note: Ratio of xylene/ paraffin wax= 1:1

Gentle agitation improves the penetration of the ‘xylene + paraffin wax’ mixture in tissue)

- Liquid paraffin wax: Incubate overnight/ 16-18 hours at 60ºC.

(Note: use paraffin wax with melting temperature 56-58ºC. For incubation in liquid paraffin wax, keep the tissue in an incubator set at a temperature of 62-64ºC. That’s how the temperature of liquid paraffin wax will reach 60º. Gentle agitation improves the penetration of paraffin wax in tissue).

Step 3. The casting of tissue wax blocks

- The cassette was opened to view the tissue sample and choose a mold that best corresponds to the size of the tissue.
- Molten paraffin was placed in the mold. Tissue was transferred into the mold using warm forceps, placing cut side down.
- The mold was transferred to a cold plate, and gently press the tissue flat.
- The labeled tissue was placed cassette on top of the mold as baked and the tissue was in the desired orientation. Insist forcefully. To the mold, add hot paraffin wax.
- In 30 minutes, paraffin solidifies. Pop the paraffin wax blocks out of the mold.

Step 4. Microtomy

- The water bath's temperature was set at 50˚C. The wax blocks were placed and faced downwards on the melting ice cube for two minutes.
- The wax blocks were aligned vertically and faced the blade as insert the blocks into the microtome chuck.
- The blocks were trimmed, and tissue portions were cut at 10mm intervals. The optimal setting for the blade's angle was between 5 and 15 degrees celsius.
- The blocks were ribboned successfully, and the section was cut into another four portions, pick them up with forceps or a fine paintbrush, and floated them on top of the water bath at 50°C.
- Float the parts on the glass slides' shiny surface. [pre-coated with Mayer’s albumin (50% egg albumin + 50% glycerol)]
- The slides were placed with paraffin sections at 65ºC incubator/oven for 20 minutes (so the wax just starts to melt) to bond the tissue to the glass slide (or the slides were kept with paraffin sections at room temperature overnight).
- Slides were stored overnight at room temperature.

Step 5. Deparaffinization

- Slides were transferred to the xylene bath and run three xylene changes for five minutes each.
- Excess liquid was removed and rehydrated slides were two changes of fresh absolute ethanol for 10 min. each.
- Excess liquid was removed and slides were placed in fresh 95% ethanol for two changes at 10 min. each.
- The slides were placed in deionized water for 1 min.

Step 6. Antigen Retrieval - Unmasking of Antigen

- The slides were placed in a petridish and treated with sodium citrate buffer at 95 degrees celsius for two changes at 5 minutes each.
- The slides were cooled at room temperature and the slides were washed 3 times for 2 min. and excess liquid was aspirated with tissue paper.

Step 7. Primary and secondary antibody reaction

- 0.1% Triton X-100 was added for 10 min. on the slide and washed slide 3 times with deionized water.
- Excess liquid was aspirated with tissue paper. Further steps were taken under the humidity chamber.
- Peroxidase block was added on the slide for 5 min. and washed the slide 3 times in deionized water.
- The tissue with slide was pre-incubated with 5% BSA for 2 hours at room temperature and the slide was washed 3 times with phosphate buffer saline.
- (1:1000) diluted TNF-alpha primary antibody was added on the slide for 4˚c overnight.
- A FITC conjugate secondary antibody that had been diluted 1:500 and was put on the slide and left there for 1 hour at room temperature and the slide was washed with phosphate buffer saline. On the slide, the DAB substrate was applied.

Step 8. Hematoxylin & Eosin (H&E) staining

- Xylene : 3 changes of 5 minutes each
- 100% ethanol : 2 changes of 5 minutes each
- 95% ethanol : 2 changes of 1 minute each
- 75% ethanol : 1 minute
- 50% ethanol : 1 minute
- Milli-Q water : 10 minutes (minimum)
- Gill’s Hematoxylin 2.0 : 30 minutes
- Tap water : 60 minutes
- Eosin alcoholic : 10 to 30 dips
- 75% ethanol : 30 dips
- 100% ethanol : 3 changes of 1 minute each
- xylene : 3 changes of 1 minute each
- Mount in DPX mounting medium and cover slip.

CHAPTER 5

RESULTS AND DISCUSSION

RESULT

1. EXTERNAL OBSERVATORY PARAMETERS

1.1 Body Weight

Animals exposed to different doses of ionizing radiation followed by dermal wounds were observed for change in body weight daily. Rats were divided into 6 treatment groups: Rats (n= 6), which were given sham irradiation, excision wound alone, (5Gy and 6Gy) radiation alone, and (5Gy and 6Gy) radiation+ excision wound and observed for change in body weight on 0,7, 15, and 30 days. Animals exposed to Sham irradiation and excision wound alone that did not induce any body weight loss. Radiation alone rats decreased the body weight till day 7 as compared to SI and EW. Whereas CI rats showed the significant reduction in body weight till day 10 and thereafter the body weight gain was much slower than other treatment groups. In 6Gy + EW group the body weight loss was more as compared to 5Gy + EW.

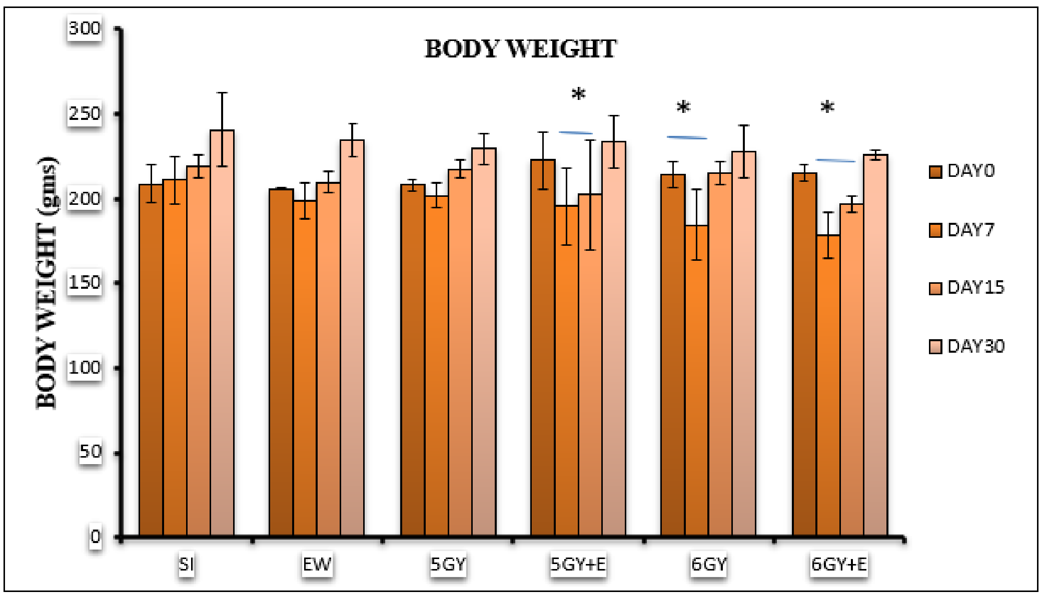


Figure 18: Effect of combined injury (CI) on the body weight of rats at different time intervals. N= 6 per group; * significantly different in comparison to SI and EW at P< 0.05.

1.2 Wound Area

SD rats were exposed to combined radiation injury. Irradiation was followed within 1 hour by dorsal skin excision wound injury. Rats were divided into 3 treatment groups, that was excision wound alone, and (5Gy and 6Gy) radiation+ excision wound. The wound area was observed and noticed on day 0 then observed at 7, 15, and 30 days. After the 15 days of study, wound closure was observed and significant hair growth and scar formation were observed in the EW group. While in 5Gy+E and 6Gy+E the wound healing was significantly altered on the observed days. The delay in wound healing in CRI groups indicates the synergistic effects of radiation and wound on the rats which possibly can lead to morbidity and mortality.

Table 6: The table depicts the significant changes in wound healing.

GROUPS	DAY0	DAY7	DAY15	DAY30	PERCENTAGE WOUND REDUCTION
EW	459.5 ± 95.45	262.5 ± 81.3	41.5 + 6.36	4.5 ± 0.70	99
5GY+EW	415.63 ± 154.07	315.11 ± 150.18	101.1 ± 143.14	24.6 ± 18.6	94.07
6GY+EW	466.66 ± 42.90	227.25 ± 47.11	49.66 ± 12.5	38.6 ± 1.52	91.7

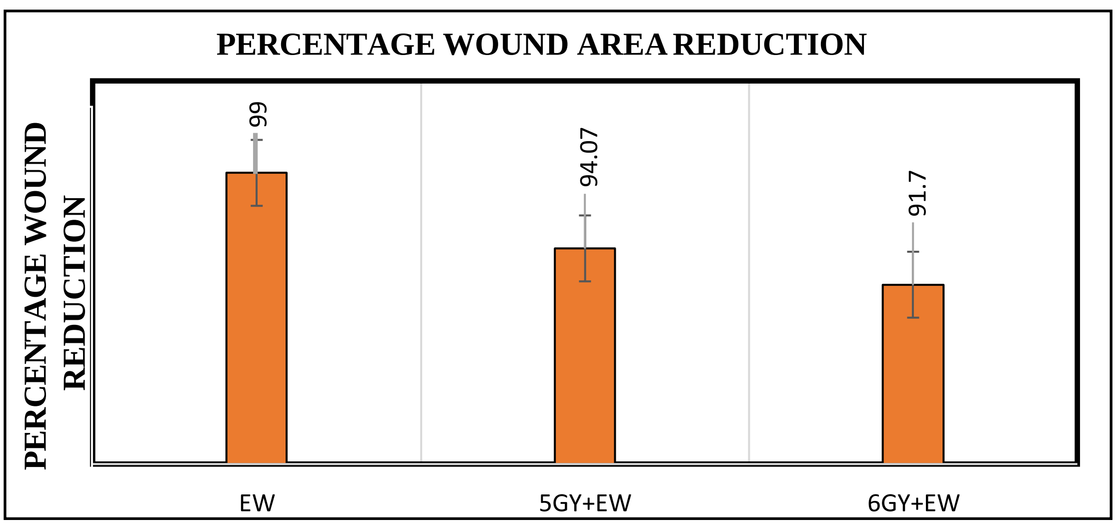


Figure 19: Effects of percentage of wound reduction in rat skin to the comparison of excision wound alone, the CI groups of SD rats N= 6 per group were observed.

1.2 Wound Score

Wound scoring was done by determining the wound appearance. A wound score was evaluated by observing the presence or absence of black eschar, odema/swelling, fluid discharge, appearing of pus, and bleeding from the wound. In EW group, only eschar formation was seen on day 7. In 5Gy + EW, eschar appeared on day 7 whereas the mild fluid discharge and pus formation was observed on day 15 group. In 6Gy + EW, eschar and mild fluid discharge appeared on day 7 whereas the pus formation was observed on day 15 group.

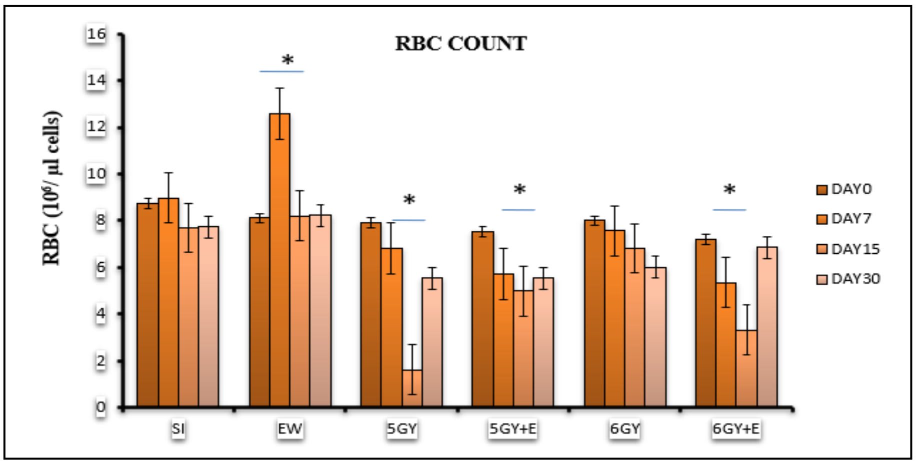
Table 7: The table depicts the wound score over the duration of 30 days.

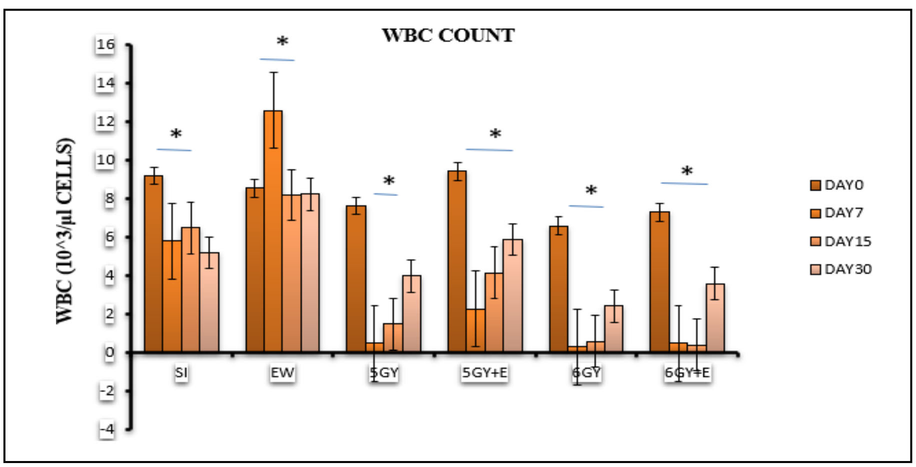
FEATURE OF WOUND BED	GROUP	DAY0	DAY7	DAY15	DAY30
Eschar formation	EW	0	1	0	0
Odema		0	0	0	0
Fluid discharge		0	0	1	0
Appearance of pus		0	0	0	0
Bleeding from wound		0	0	0	0
Eschar formation	5Gy +EW	0	1	0	0
Odema		0	0	0	0
Fluid discharge		0	0	1	0
Appearance of pus		0	0	1	0
Bleeding from wound		0	0	0	0
Eschar formation	6Gy +EW	0	1	0	0
Odema		0	0	0	0
Fluid discharge		0	1	0	0
Appearance of pus		0	0	1	0
Bleeding from wound		0	0	0	0

2. <u>INTERNAL OBSERVATORY PARAMETERS:</u>

2.1 Hematology data

A hematological evaluation of blood was done to study the change in population. The size of various cellular components of blood gets altered due to gamma irradiation, which reflects the state of the hematopoietic system functioning when compared with the control. RBC, WBCs, and platelets were significantly decreased in 5Gy, 5Gy+EW, and 6Gy, 6Gy+EW groups. Radiation causes hematopoietic damage and the total blood count in both radiation alone and combined radiation injury groups. The significant changes were observed in WBC count in all treated groups on d0, d7, d15, and d30. RBC count was not much altered but, in some groups, a significant decrease was observed post-day 10. Platelet count was high in EW alone on day 7 which indicates the homeostasis phase of wound healing while it was low for other treated groups. Whereas, the significant decrease in WBCs on days 7, 15, and 30 the data demonstrated the loss of certain precursor stem cells in bone marrow due to gamma irradiation.





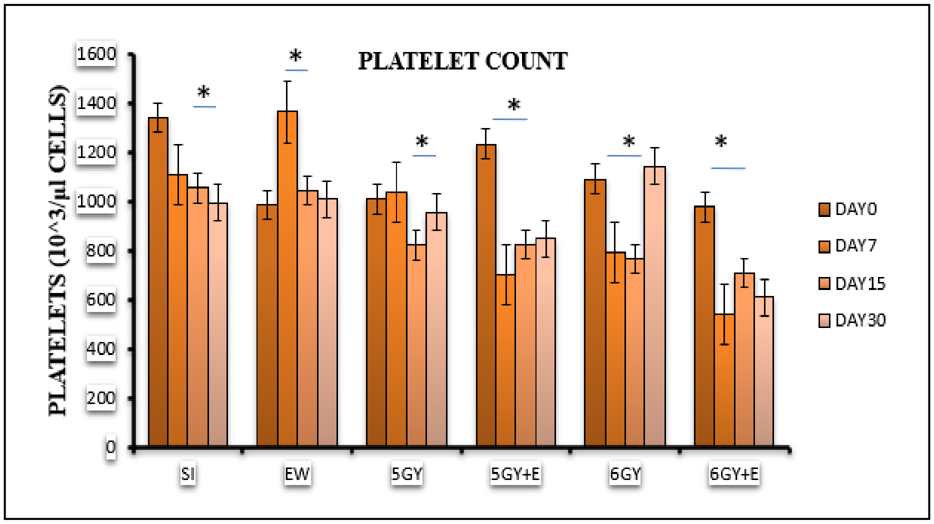


Figure 20: Effect of CI on hematological changes in rats at different time intervals. N=6 animals in each group. *$p < 0.05$ for radiation alone and RCI vs. sham and EW.

2.2 Estimation of differential leucocyte count

The DLC was estimated to check the changes in the distribution of leukocytes. It was observed that the lymphocytes% of excision wound alone and all treated groups were significantly changed on d7, d15 and d30 in comparison of sham. Whereas, granulocytes% on d7 were increased but on d15 and d30 shows the significantly changes in all treated groups

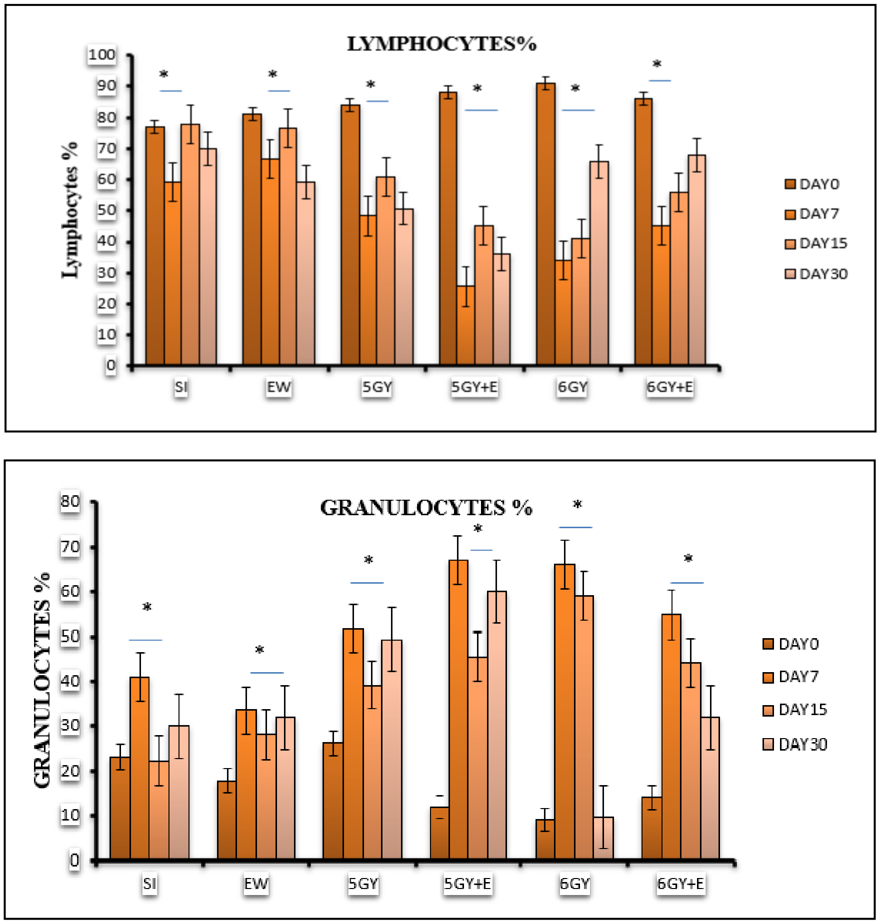


Figure 21: Effect of CI on granulocytes% and lymphocytes% changes in rats at different time intervals. N=6 animals in each group. *$p < 0.05$ for radiation alone and RCI vs. sham and EW.

2.3 Blood culture data analysis

The growth of bacteria was shown on the nutrient agar medium, after all, dilutions of blood with 0.9% saline and CFU was counted of 5Gy with excision wound and 6Gy excision wound (data as shown below). The appearance of colonies was observed on the nutrient agar medium.

Table 8: The table represents CFU counts per ml for different treated groups at different time points. The high CFU/ml indicates sepsis or infection in the treated groups.

GROUPS	DAY0	DAY7	DAY15	DAY30
5R	0	$7\times10^3\pm1.4\times10^3$	$2.5\times10^3\pm0.7\times10^3$	0
5RE	0	$20.5\times10^3\pm3.5\times10^3$	$14\times10^3\pm2.8\times10^3$	$7\times10^3\pm1.4\times10^3$
6R	0	$15\times10^3\pm2.6\times10^3$	$24\times10^3\pm4.2\times10^3$	0
6RE	0	$55\times10^3\pm10.6\times10^3$	$36\times10^3\pm9.8\times10^3$	$10\times10^3\pm2.1\times10^3$

2.4 Bone Marrow Analysis

2.4.1 May Grunwald Staining

Post irradiation in rats, significant decrease in the bone marrow cell count in all treated groups on d30 was observed. The femur bone marrow cells were stained with May Grunwald and giemsa stain which were subsequently examined for the presence of PCE and NCE. The fraction of immature and mature cell population was calculated. The decrease in ratio determines the erythroblast cytotoxicity in bone marrow. Normally an equal number of PCEs and NCEs are present in the bone marrow but during damage conditions the PCE and NCE count decreases in comparison to sham. A total of 1000 PCE and NCE were counted and the ratio was determined. The treated groups indicate erythroblast cytotoxicity in bone marrow on day30 post irradiation.

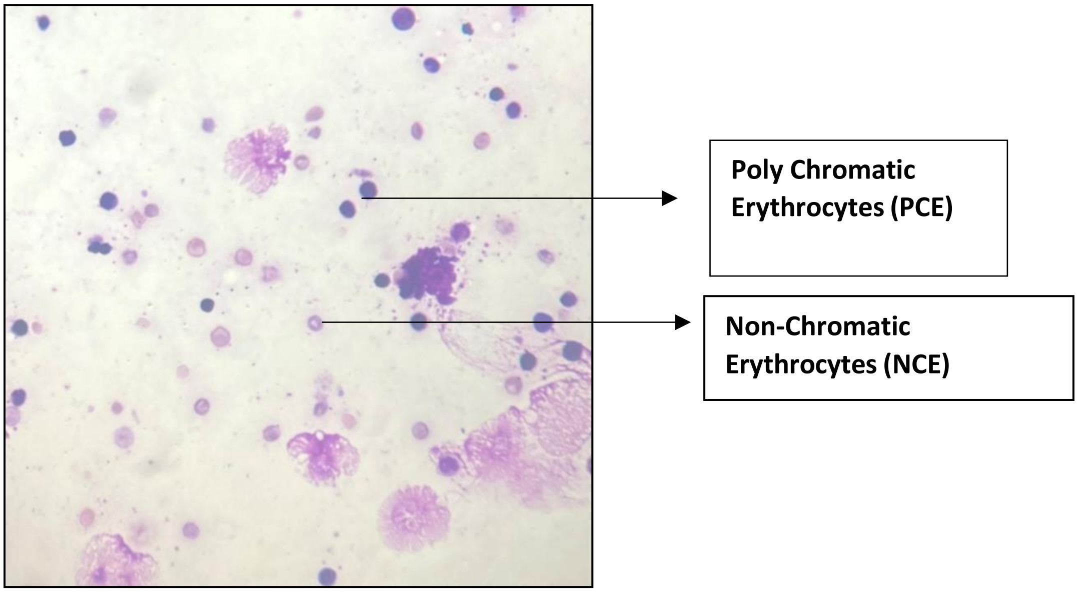


Figure 22: The representative image shows the number of PCE and NCE bone marrow cells at 100x magnification.

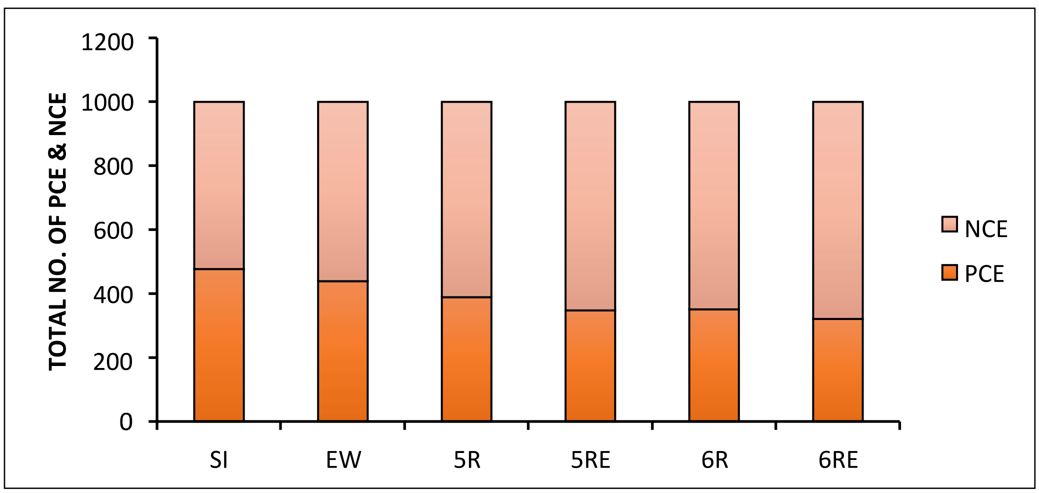


Figure 23: The figure shows the difference in the number of PCE and NCE bone marrow cells count of all treated groups in comparison to sham. Whereas, the treated groups indicate erythroblast cytotoxicity in bone marrow on day30 post-irradiation.

2.4.2 Apoptosis detection by acridine orange and ethidium bromide dual fluorescent staining

The dual fluorescent acridine orange and ethidium bromide (AO\EB) assay was performed for the observation of morphological apoptosis including chromosome condensation on bone marrow. In this assay, AO enters viable cells and nonviable cells and emits green or red fluorescence when binding to DNA and RNA, respectively: EB enters on viable cells and emits orange and red fluorescence when binding to DNA and RNA, respectively hence, normal and apoptotic nuclei emit bright orange fluorescence in non-viability cases. Five hundred cells were examined per animal under a fluorescent microscope at 400X magnification with the blue excitation filter 480 nm and yellow emission barrier filter 515 – 530 nm. The apoptotic index was calculated according to the following formula = [no of apoptotic cells/total cell count] * 100.

The figure depicts the induction of morphological apoptotic events in bone marrow cells of SD rats of different groups at day 30. The apoptotic index in radiation and combined radiation injury groups is used as an indicator of membrane integrity to assess apoptosis/cytotoxicity in whole nucleated bone marrow cells. The damage in RA and RCI groups is evidenced by increased apoptotic rate.

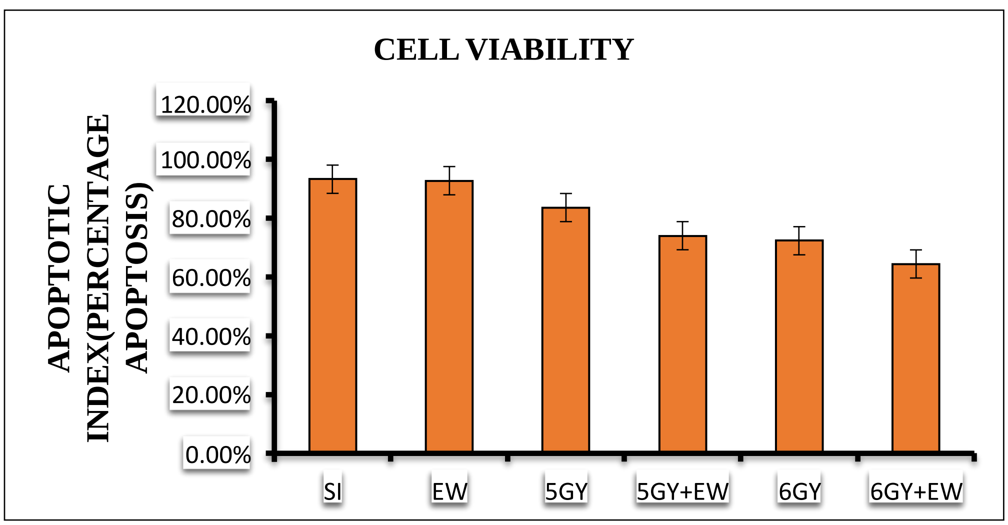


Figure 24: The figure depicts the induction of morphological apoptotic event in bone marrow cells of SD rats of different groups at day30. Whereas, the percentage of cell viability were decreased in all treated groups in comparison to sham.

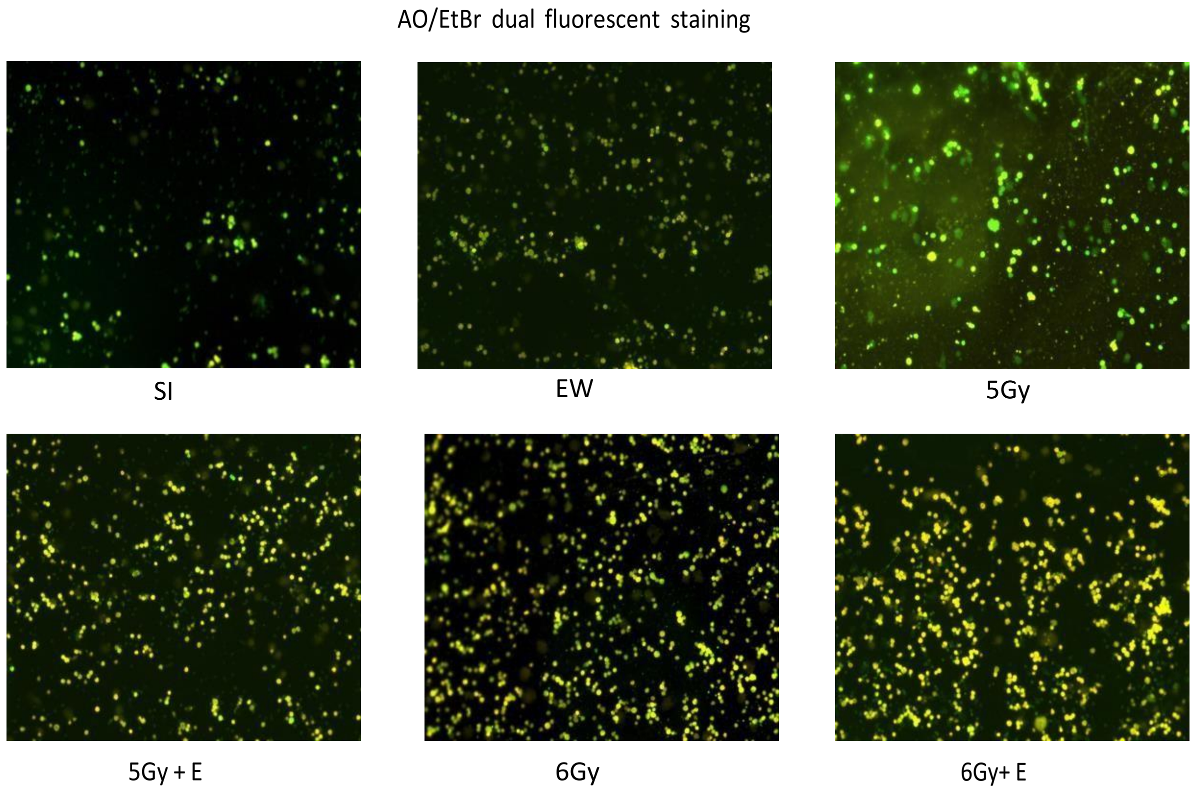
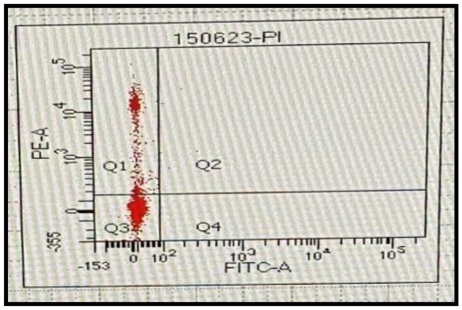


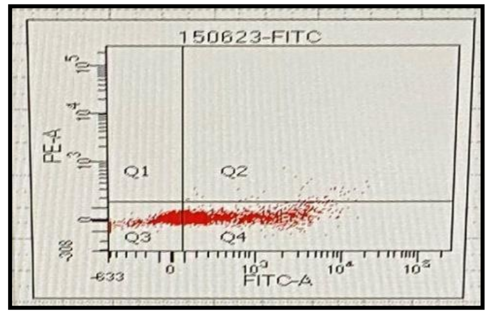
Figure 25: The representative image shows the viable and non-viable cells after staining with acridine orange/ethidium bromide dual fluorescent staining. The bright green nuclei indicate the viable cells with intact membranes; the bright green condensed nuclei indicate early apoptotic cells and the yellow condensed nuclei indicate the late apoptotic cells or non-viable cells.

2.4.3 Apoptotic Cell Detection Using Annexin V-FITC:

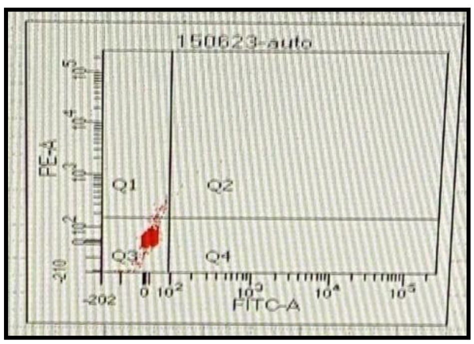
It is a prevalent procedure to use the calcium-dependent phospholipid-binding protein annexin V as a marker to distinguish apoptotic cells. The interaction between Annexin V and externalized phosphatidylserine on the surface of apoptotic cells is what drives the Annexin V-FITC test. A fluorochrome called FITC is coupled with Annexin V. FITC-labeled Annexin V binds to exposed PS and distinguishes apoptotic cells from healthy ones. Our results imply that rats exposed to radiation exhibit high levels of Annexin V-FITC binding (green fluorescence) as a result of increased phosphatidylserine (PS) externalization as compared to control rats, suggesting that animals exposed to radiation speed up the apoptotic process.



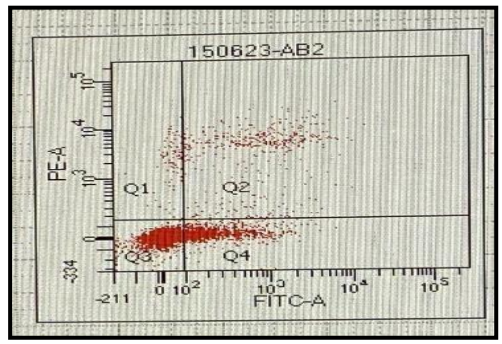
(a)



(b)



(c)



(d)

Figure 26: Representative images of FACS analysis staining a) Cells stained only with PI, b) Cells stained only with Annexin V-FITC c) only cells d) Cells stained with annexin V and FITC.

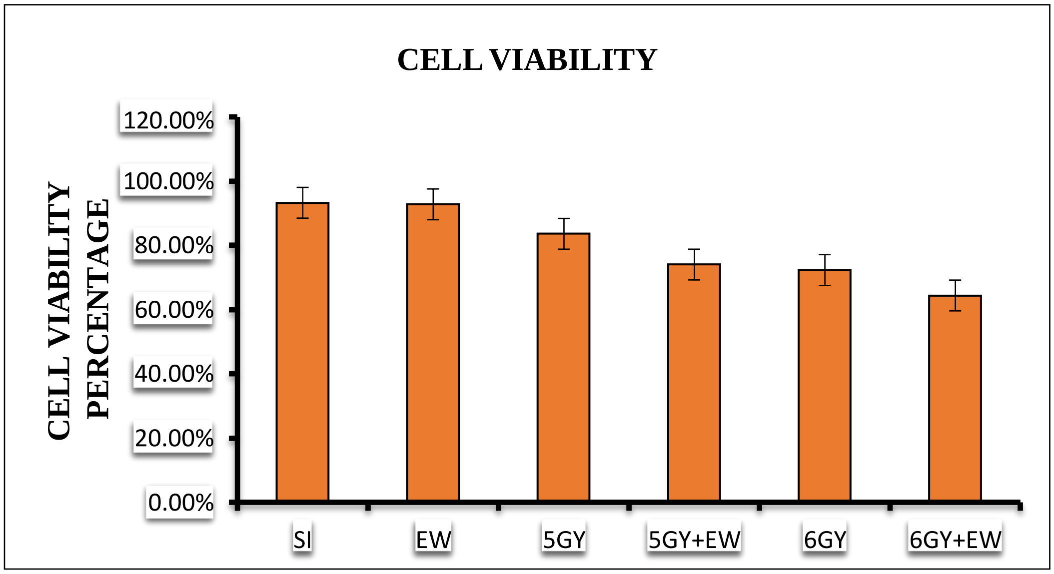


Figure 27: Percentage of cell viability assessed by annexin-v and PI staining.

Annexin V –PI staining (Immunofluorescence)

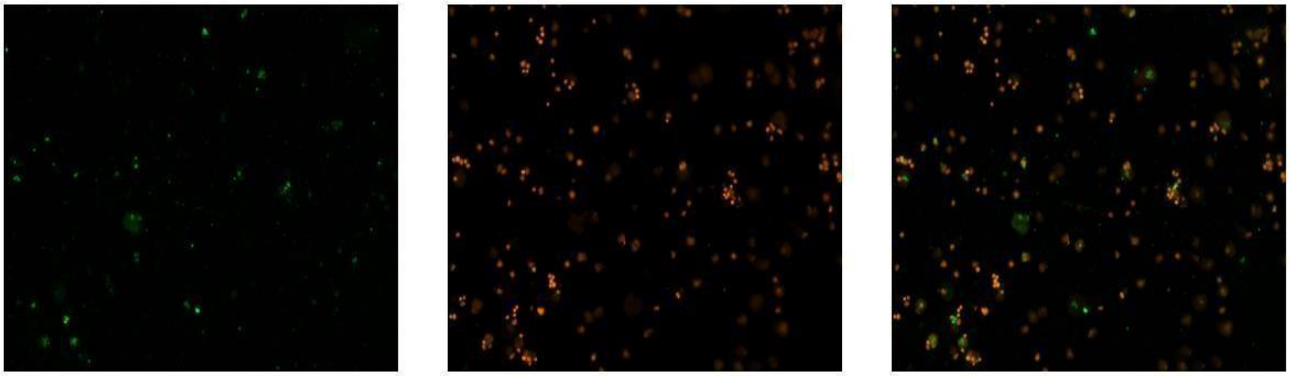


Figure 28: Microscopic evaluation of Annexin-V FITC representing the percentage of cell viability assessed by annexin-v and PI staining.

3. HISTOLOGY

To evaluate the histopathological changes in the skin of Sprague Dawley rats; rats were dissected on the 30th day and then were stained with H&E and slides were prepared for the histological examination. Histopathology study showed the infiltration of inflammatory cells to the damaged site and the morphology of the skin wound. Control skin showed the normal structure of the epidermis with regularly distributed skin layers and cells. In treated groups, the damage was prominently seen with the widening of intracellular spaces, the significant decrease in dermal thickness, adipose tissue integrity, and hair follicles intensity. On day 30 (RA and CRI both) the epidermal cells within the wound are separated, and the cellular spaces widens and fibroblast cells were proliferating in comparison to simple wound group. Also, abnormal cellular nuclei and chromatin clumping were observed in comparison to excision wound alone.

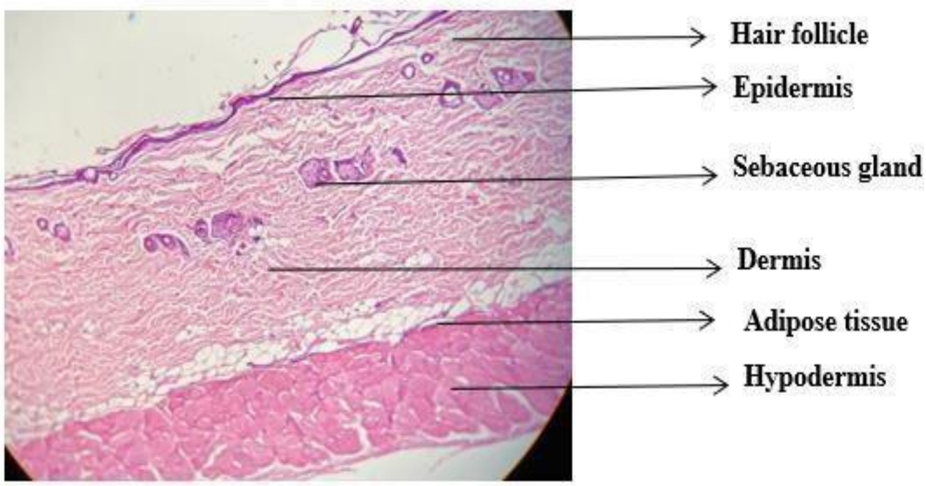
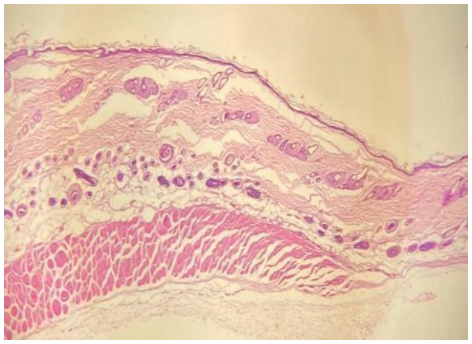
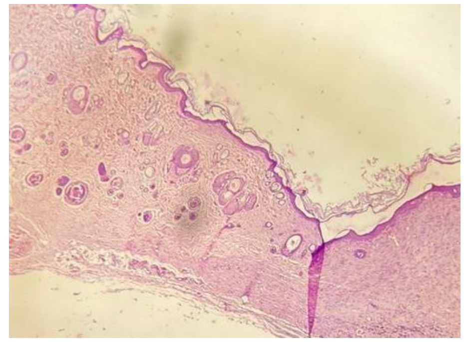


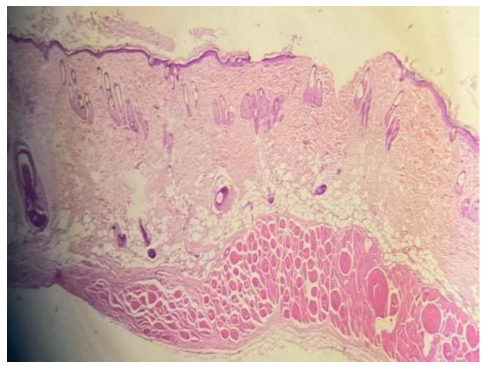
Figure 29: Microscopic observations of H&E stained rat skin section of SI group with no abnormalities.



5Gy+E



6Gy+E



EW

Figure 30: Representative images of H&E sections of skin wound scar at day 30 a) EW c) 5Gy+E c) 6Gy+E showing abnormalities in the skin integrity.

4. **BIOCHEMICAL PARAMETERS**

The levels of MDA and GSH were estimated. RA and RCI groups showed a significant increase in levels of MDA (1.5-2.5 folds) with a significant decrease in the level of GSH in comparison to SI or EW. RCI groups when compared to RA also show significant differences indicating oxidative stress condition at d30 post irradiation and wounding.

4.1 MDA (Malondialdehyde) Estimation

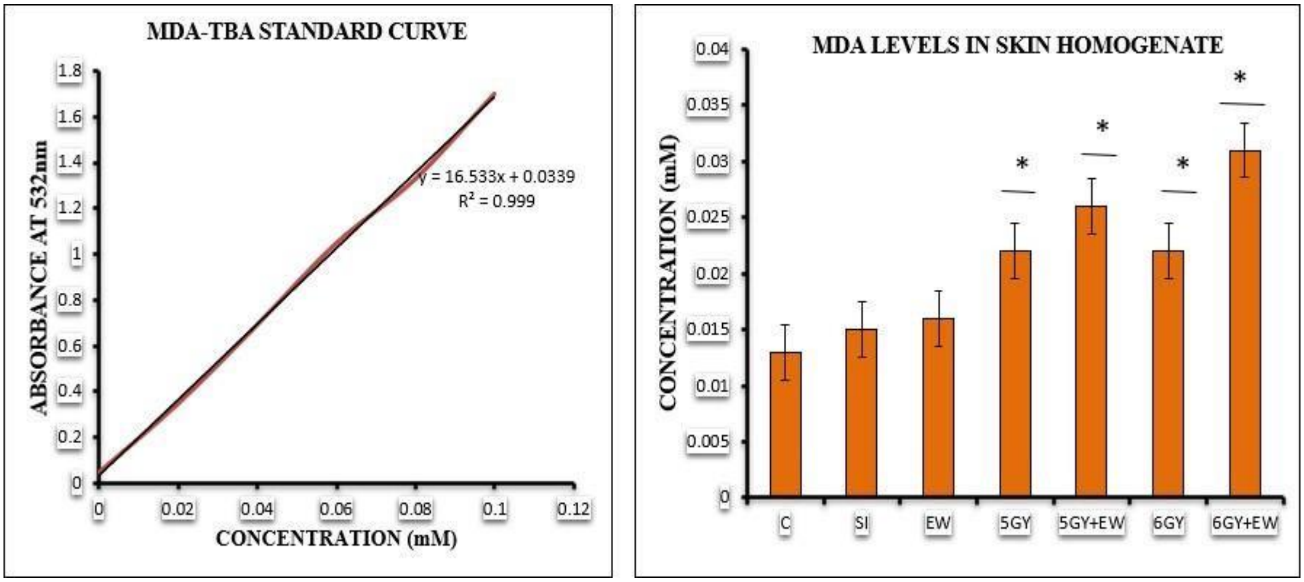
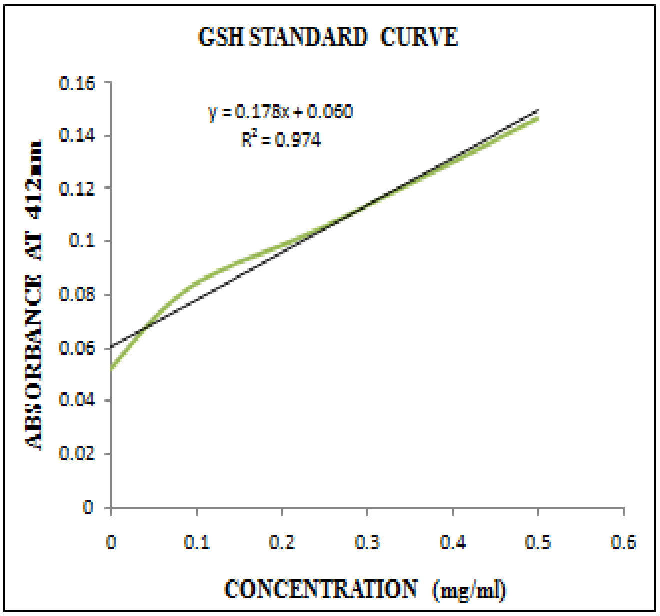


Figure 31: The figure a) Shows the standard curve of MDA-TBA at 532nm b) MDA levels in skin homogenate with the concentration (mM) of all groups. The skin MDA levels of RA and RCI groups were found to be significantly higher than those of SI or EW group. Whereas, symbol* shows the significant changes * $p < 0.005$ for radiation alone and RCI vs. sham and EW and also increase in MDA concentration indicates oxidative stress in RCI groups on day30 post irradiation and wounding.

4.2 GSH (Reduced Glutathione Estimation)



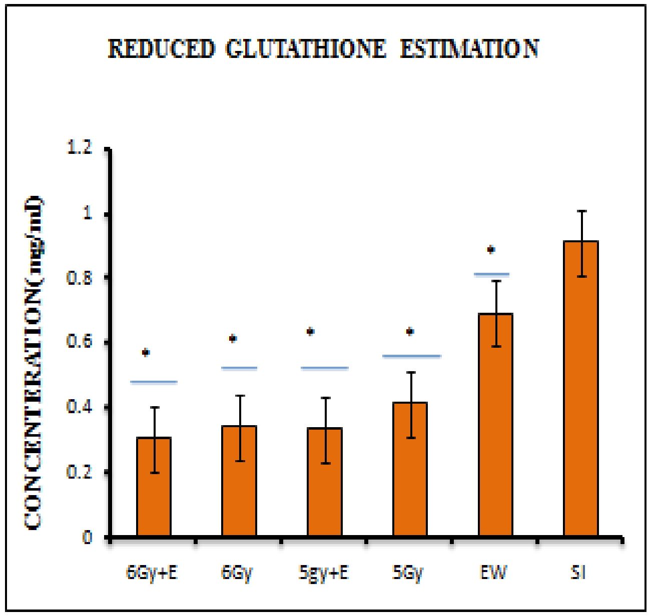


Figure 32: The figure a) Standard curve for Reduced glutathione at 412nm b) GSH concentration in skin homogenate. In treated groups; there is a substantial decrease in GSH levels in EW, RA, and RCI groups. Whereas, N=6 animals in each group shows the significant changes * $p < 0.005$ for radiation alone and RCI vs. RA, sham and EW.

5. MPO ASSAY

The presence of neutrophils infiltrates in the skin samples was confirmed by analyzing the activity of MPO in skin homogenate. MPO activity in RA and RCI groups was significantly increased as compared to SI.

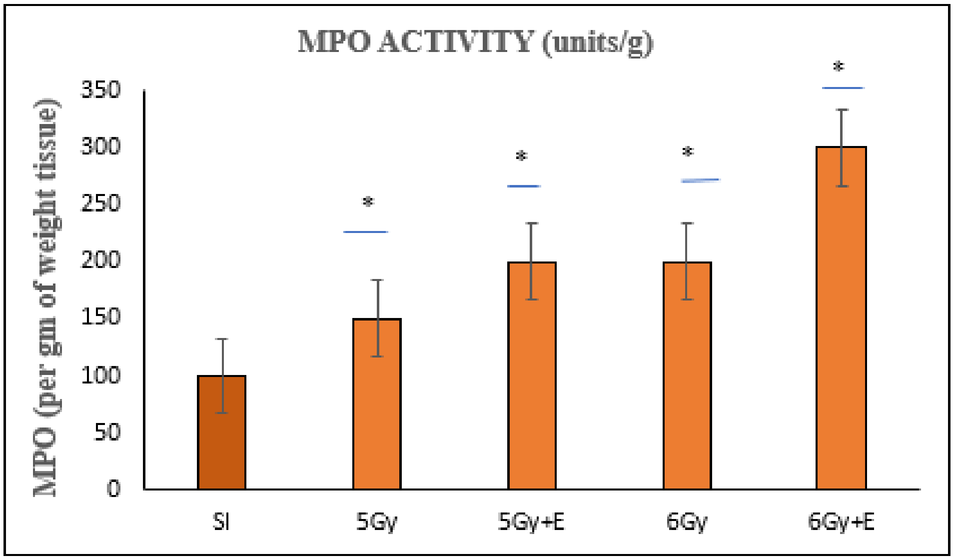


Figure 33: The figure determine the MPO activity when compared with sham. MPO an inflammatory marker is elevated in both RA and RCI that indicates inflammation on day 30 post irradiation. Whereas, N=6 animals in each group shows the significant changes * $p < 0.005$ for radiation alone and RCI vs. RA, sham and EW.

6. ESTIMATION OF INTERLEUKINS IL-1 β, IL-6 and IL-8 SANDWICH ELISAASSAY

The interleukins (IL-1 β, IL-6, and IL-8) were estimated through the kit of BT lab sciences. Interleukins are a group of cytokines that were first seen to be expressed by white blood cells at the inflammation site at the time of injury. Radiation exposure induces cytokine production in the surviving rats of sham, excision wound alone, (5Gy and 6Gy) radiation alone, and (5Gy & 6Gy) radiation with excision wound alone. Therefore, the levels of cytokines in the rats sample collected on day 30 after irradiation from surviving animals in all groups were determined. Compared to the sham irradiated rats, three cytokines IL-1 β, IL-6, and IL-8 were significantly elevated in samples from wound sites of RCI rats exposed to 5Gy and 6Gy dose rate.

6.1 IL-1Beta

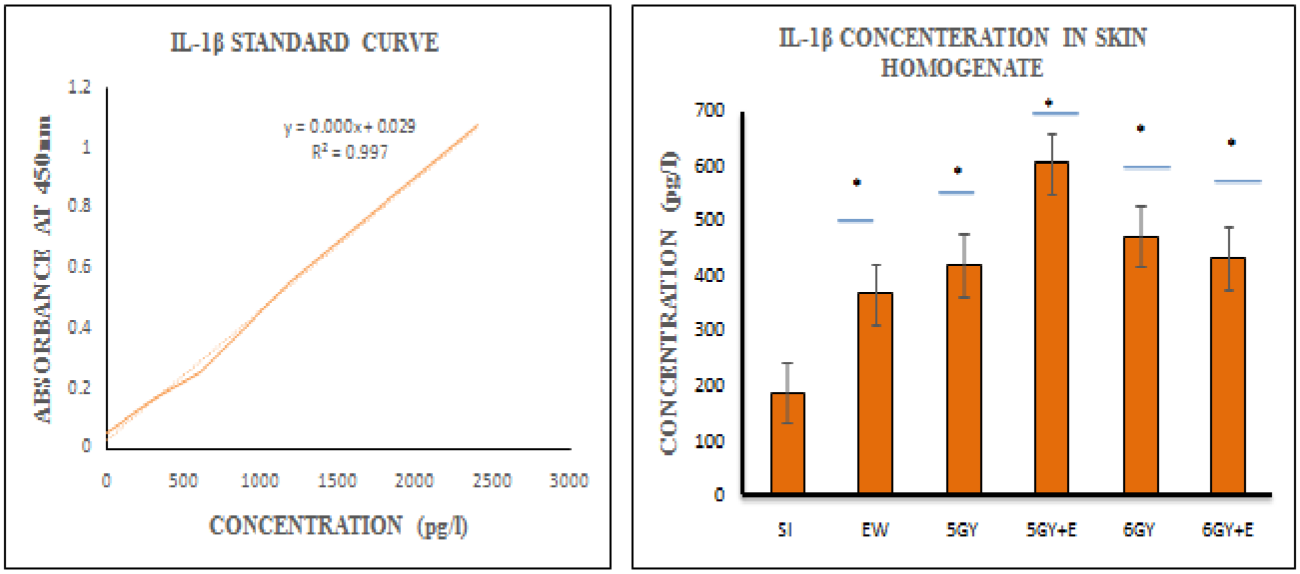


Figure 34: The figure a) shows the standard curve of IL- 1Beta at 450nm. b) IL- 1Beta proinflammatory cytokine levels in skin homogenate with the concentration (mM) in all groups were observed. The levels of RA and RCI groups were found to be significantly higher than those of SI group. (Where symbol* shows the $p<0.05$ vs. SI) the EW, 5Gy+EW, 6Gy+Esignificant increase in IL-1Beta concentration of RA &RCI treated groups on day 30 post-irradiation and wounding.

6.2 IL 6

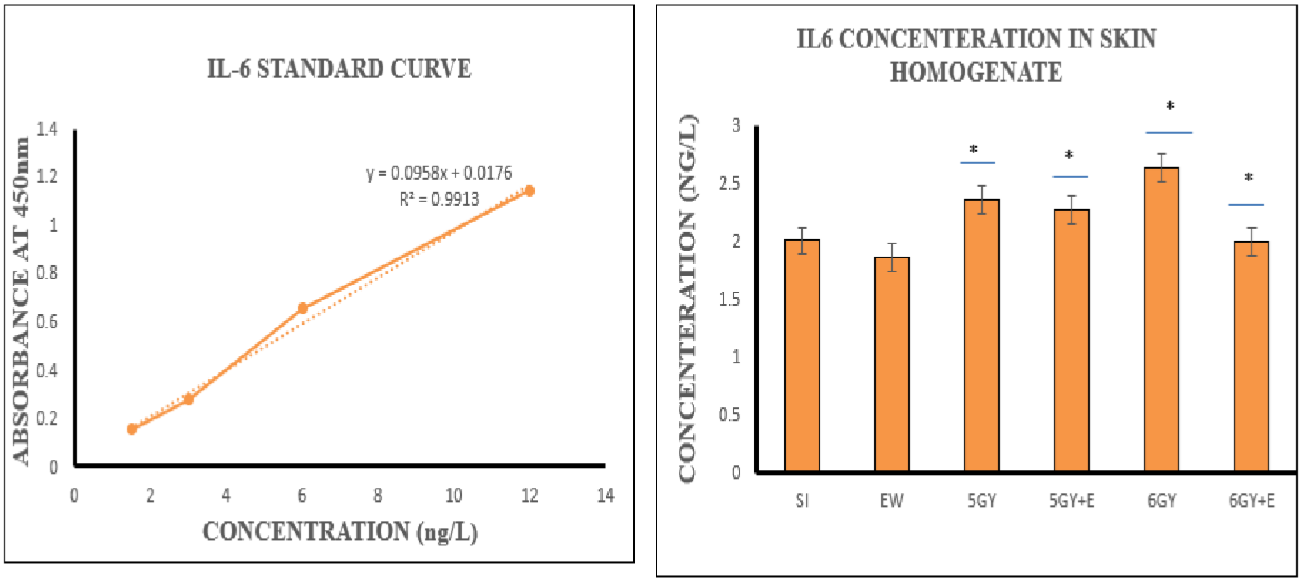


Figure 35: The figure a) shows the standard curve of IL- 6 at 450nm. b) IL- 6 proinflammatory cytokine levels in skin homogenate with the concentration (mM) in all groups were observed. The levels of RA and RCI groups were found to be significantly higher than those of SI group. (Where symbol* shows the $p<0.05$ vs. SI & EW) of RA 5Gy, 6Gy and 5Gy+EW, 6Gy+Eshows the significant level in IL-6 concentration of RA &RCI treated groups on day 30 post-irradiation and wounding.

6.3 IL8

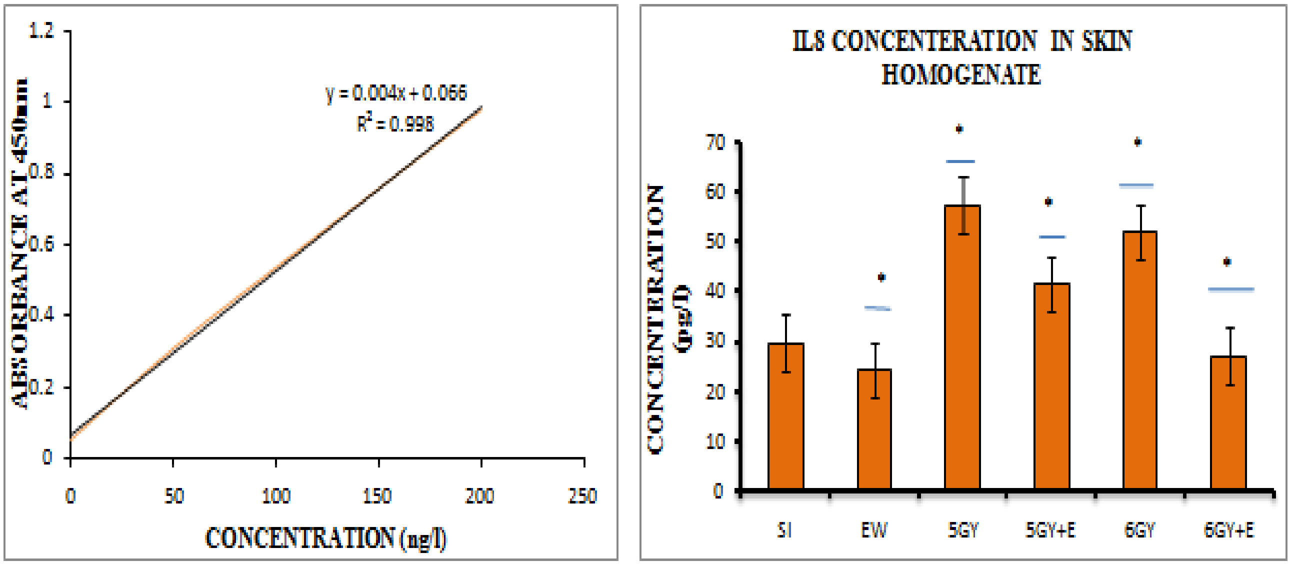
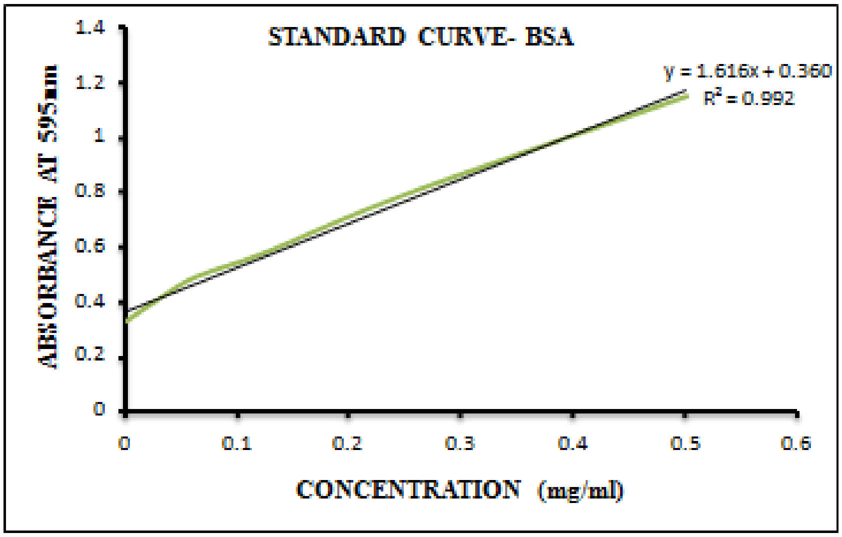


Figure 36: The figure a) shows the standard curve of IL-8 at 450nm. b) IL-8 proinflammatory cytokine levels in skin homogenate with the concentration (mM) in all groups were observed. The levels of RA and RCI groups were found to be significantly higher than those of SI group. (Where symbol* shows the $p<0.05$ vs. SI) of EW, RA 5Gy, 6Gy and 5Gy+EW, 6Gy+E shows the significant level in IL-8 concentration of RA and RCI treated groups on day 30 post-irradiation and wounding.

7. <u>UNKOWN PROTEIN QUANTIFICATION ASSAY</u>

The stock solution of BSA (1 mg/ml) was prepared to form different concentrations by serial diluting the stock solution. The Bradford reagent was added to the protein sample in 1:1 ratio. Absorbance was recorded at 595 nm.



GROUPS	OD AT 595nm	BLANK NORMALISED	CONCENTRATION FROM GRAPH (mg/ml)
Blank	0.33	0	
Control	1.733	1.403	0.645420792
SI	1.637	1.307	0.586014851
EW	1.575	1.245	0.547648515
5GY	1.945	1.615	0.776608911
5GY+E	1.632	1.302	0.582920792
6GY	1.51	1.18	0.507425743
6GY+E	1.418	1.088	0.45049505

Figure 37: Representative image and shows the standard curve of BSA concentration at absorbance 595 nm and table depicts the standard BSA concentration of all groups.

SDS PAGE – Coomassie Staining

SDS-PAGE is a reliable technique for estimating the molecular weight (MW) of an unknown Same concentration of protein (30mg/ml) of different groups was loaded into each well and after the run the gel was stained with coomassie to visualize the separated bands.

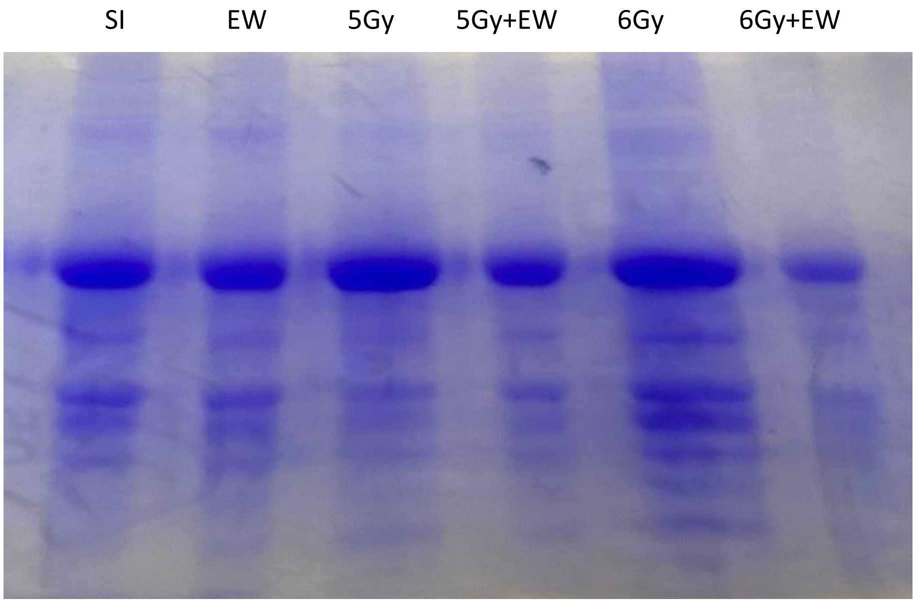


Figure 38: The representative image shows the SDS PAGE of proteins with same concentrations and stained with Coomassie brilliant blue.

CHAPTER 6

CONCLUSION

Conclusion

For the development combined radiation injury, SD rats were irradiated with ^{60}Co-irradiation at doses of (5 Gy and 6 Gy) followed by skin wound damage. Radiation combined with wounding increased the mortality, body weight loss, reduction of white blood cells, and platelets along with retarded wound healing and the effect was radiation dose dependent. Radiation combined with wound trauma results in decrease in lymphocytes, macrophages, neutrophils, platelets, cell adhesion molecule, tissue integrity and stem cells but increases in the activity of the reactive oxygen species. Also the apoptosis were observed more in 6Gy+EW as compared to 5Gy. To improve survival, countermeasures need to be investigated that shorten wound closure time and improve survival. The aim of our work is to develop CRI injury in animals so that countermeasures can be tested against CRI.

CHAPTER 7

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