Project report

on

Effect of Hypoxia on Tight Junction Proteins in Intestine

Submitted in Partial Fulfilment of the Requirement for the Degree of M.Sc. Biochemistry

Submitted by

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CANDIDATE DECLARATION

I hereby declare that the dissertation entitled "Effect of Hypoxia on tight junction proteins in Intestine" submitted by me in partial fulfillment for the degree of M.Sc. in Biochemistry to the Division of Biochemistry, School of Basic and Applied Science, Galgotias University, Greater Noida, Uttar Pradesh, India is my original work. It has not been submitted in part or full to this University of any other Universities for the award of diploma or degree.

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List of Abbreviations

S.No.	Short Form	Abbreviation
1.	TNF	Tumor Necrosis Factor
2.	IL	Interleukin
3.	TIRs	Toll Like Receptors
4.	LPS	Lipopolysaccharides
5.	TJ	Tight Junction
6.	ZO	Zonula Occludens
7.	CLDN-1	Claudin-1
8.	IBD	Inflammatory Bowel Disease
9.	HIF	Hypoxia Inducing Factor
10.	IEC	Intestinal Epithelial Cell
11.	HRE	Hypoxia Response Element
12.	DMSO	Dimethylsulphoxide
13.	SD	Sprague Dawley
14.	NFkB	Nuclear factor kappa beta

ABSTRACT

The present study involves the study of effect of hypoxia on tight junction proteins in intestine in Male Sprague Dawley (SD) rats. Simulated hypobaric hypoxia chamber (Matrix, India) was used to produce hypoxic conditions. Animals were kept in the chamber for 6h at an altitude of 7,620m (280mm Hg) with sustained temperature of 25±2˚C. After exposure, animals were sacrificed and blood was removed by perfusion with cold PBS. The intestine tissue was collected, washed with cold saline (0.9% NaCl) and further biochemical analysis was performed. The biochemical parameters like malonaldehyde, reactive oxygen species (ROS) and reduced Glutathione (GSH) in intestine were determined. The data was analyzed using graph pad prism software. The result indicates that reactive oxygen species (ROS) and malonaldehyde level were increased and glutathione level were decreased after hypoxia ($p<0.5$) while the reverse effect was observed after introduction of Quercetin in hypoxic condition.

INTRODUCTION

Hypoxia in broad sense affect tight junction including loss of barrier function and electrical resistance of epithelium. The permeability changes are also imputable to alterations in the distribution of occludin, zonula occludens-1 (ZO-1), ZO-2, and cingulin. TJ integrity is also influenced by perturbations of the interaction with the actin-based cytoskeleton and by the degradation of membrane cytoskeletal proteins such as ankyrin and fodrin in ATP depletion models.

The use of natural anti-inflammatory products provides an attractive and safe alternative to modulate inflammatory disorders. Quercetin is used as flavonoid compound and found in various types of fruits and vegetables. It is also found in grains and leaves. Quercetin has capacity remove free radicals generated inside body and act as antioxidant enzymes.

High altitude

Regions on the earth's surface or in its atmosphere that are high above mean sea level are referred to as high altitude.

At high altitude, atmospheric pressure is lower than that at sea level. This is due to two competing physical effects: gravity, which causes the air to be as close as possible to the ground and the heat content of the air which causes the molecules to bounce off each other and expand.

High altitude ranges are followings:

- Ranges from 2500-3500 meters above sea level are considered as high-altitude while ranges from 3500-5500 meters above sea level are considered very high altitude. More than high altitude will be considered as extremely high altitude

Normoxia

The availability of normal oxygen level in tissue known as normoxia.

Hypoxia

The insufficient amount of oxygen into tissue is reflection of hypoxia. Although hypoxia in many instances is a pathological condition, imbalance in arterial oxygen concentrations can be part of the everyday physiology, for example, for the duration of hypoventilation coaching or strenuous bodily exercise. Hypoxia, hypoxemia and anoxemia can be differentiated by the fact that a state in which oxygen supply is not enough is considered as hypoxia, whereas anoxemia and hypoxemia refer to the less oxygen in blood (West JB, 2011).The condition in which there is total deprivation of oxygen furnish is known as anoxia.

In silent hypoxia, oxygen stage in blood cells and tissue can drop. The silent hypoxia was also detected in COVID 19 patient and reflects acute respiratory distress (ARDS) and organ failure (Levitan et. al., 2020).

Causes

Oxygen passively diffuses in the lung alveoli in accordance to a stress gradient. Oxygen diffuses from the breathed air, combines with water vapor and then to arterial blood, the region where it's partial strain is round 100 mmHg (13.3 kPa)(Kenneth B et. al, 2006). In the blood, oxygen is sufficient to hemoglobin, a protein in crimson blood cells. The binding capability of hemoglobin is influenced by using the partial stress of oxygen in the environment. A smaller quantity of oxygen is transported in answer in the blood.
Hypoxia may lead to a failure of oxygen transport to the cells at any stage at any stage. This may include reduced partial pressures of oxygen, problem with diffusion of oxygen in the lungs, inadequate reachable hemoglobin, troubles with blood flow to the give up tissue, and troubles with respiration rhythm.

Symptoms

Symptoms of hypoxia may be acute or chronic and vary in intensity from mild to severe.

- Common acute symptoms are:

  Breathing Shortness, rapid breathing, and enhanced heart rate

- Severe symptoms include:

  The inability to communicate, skin color change from blue to cherry red, dilemma, coma and

  the possibility of death.

Hypoxia may be generalized or local (Das KK et. al., 2019).

- **Localized Hypoxia-**The condition in which deficiency of oxygen occurs in a particular organ or tissue is known as localized hypoxia. Tissue not being perfused properly, may additionally ride cold and show up pale; if severe, cyanosis, a blue discoloration of the skin results as end of hypoxia. If hypoxia is very severe, a tissue may also in the end grow to be gangrenous. Extreme pain can also moreover be felt around the site.
- **Generalized Hypoxia-**The condition in which entire physique is affected and which results in a shortage of oxygen in complete body is recognized as generalized hypoxia. High-altitude cerebral edema (HACE) and high-altitude pulmonary edema (HAPE) may occur in this hypoxia (Cymerman A et.al., 1994)**.**The signs and symptoms of generalized hypoxia count number on its severity and acceleration of onset. In the case of altitude disease, the region hypoxia develops often, symptoms like numbness / tingling of extremities, fatigue, cerebral anoxia and nausea are embodied in the case of high altitude; the region hypoxia develops often (Robinson T et al., 2009). These signs are regularly difficult to determine, but early inspection of symptoms can be lethal (Bergqvist et.al, 2015).

In very fast onset of hypoxia, confusion / behavioral change/hallucinations, serious headaches / reduced level of consciousness, papilledema, breathlessness, ataxia (Robinson T et. al., 2009; Pallor et. al., 2012) tachycardia, and pulmonary hypertension at last main to the late signs and symptoms cyanosis, slow heart rate / pulmonale, and low blood pressure followed by coronary heart failure ultimately main to shock and loss of life (Hillman K et.al., 2004; Madge et.al., 2006).

On the basis of altitude, hypoxia is of two types:

- **Hypobaric Hypoxia**: It is a condition which occurs due to low level of oxygen reaching tissues at high altitude starting from 2500m above sea level.
- **Normobaric Hypoxia**: It is a condition which occurs due to low level of oxygen reaching tissues at sea level.

Classification of hypoxia:

1. **Hypoxic Hypoxia:** In this hypoxia there is a lack of oxygen in blood and lungs alveoli. This kind of hypoxia influences the physique as a complete and is one of the most serious varieties of hypoxia.
   When the PO2 of arterial blood declines, hypoxic hypoxia takes place. This might occur due to the fact stimulated PO2 is decrease than ordinary (high altitude) or it ought to be due to a respiratory hassle (e.g., hypoventilation, diffusion impairment precipitated with the aid of ventilation–perfusion mismatch, anatomic shunt of blood past the gas exchange region or pulmonary edema).
   Patient of Asthma, emphysema, pneumonia may develop hypoxia. Mechanical obstruction laryngospasm or bronchospasm may alter oxygen level from the surroundings into the lungs and cause hypoxia (Cafaro, 1960)

2. **Anemic hypoxia:** The arterial blood has sufficient oxygen in anemic hypoxia however there is a lack of functional hemoglobin. Anemic hypoxia effects on the total body. Anemic hypoxia may be result of acute or chronic hemorrhage, (Cafaro, 1960).
   Hemoglobin performs a full-size position in carrying oxygen at some stage in the body and anemia results when hemoglobin is deficient, further if tissue perfusion is also decreased than anemic hypoxia inflicts. In anemia deficiency of iron occurs very easily. As iron is used in the synthesis of hemoglobin, very less hemoglobin is synthesized when there is much less iron, due to inadequate intake or terrible absorption (Colledge NR et.al., 2010).

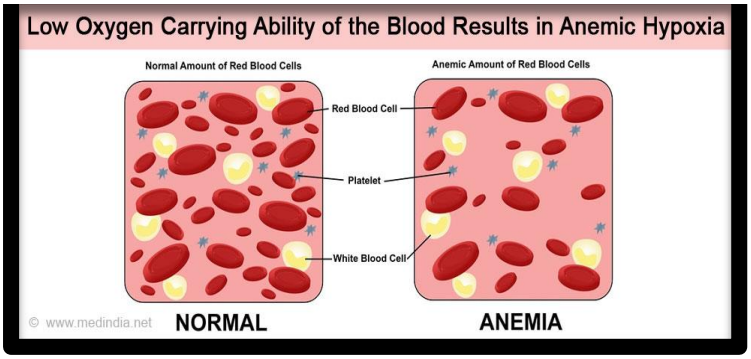

Fig. 1-Showing Anemic hypoxia

3. **Stagnant hypoxia:** Due to improper blood flow sufficient oxygen cannot reach to cells and tissues. It affects all over body.
   Sluggishness in the rate of the circulating blood permits the blood to stagnate and supply up an increased share of its oxygen. This gradual circulation additionally approves the accumulation of an increased volume of carbon dioxide in the tissues. Stagnant hypoxia is produced via failure of the circulation, impairment of venous return, and shock.

4. **Histotoxic Hypoxia:** The hypoxia occurs due to deposition of toxic compounds in tissue and blood. Cells are no longer in a position to make use of the oxygen, even though the quantity of oxygen in the blood might also be normal and under normal tension (Cafaro, 1960).
   Decline in ATP manufacturing by the mitochondria due to a defect in the cell utilization of oxygen is referred to histotoxic hypoxia. Cyanide poisoning is the main reason behind histotoxic hypoxia. The response of oxygen with cytochrome c oxidase is blocked through the presence of cyanide due to which there is drop in tissue oxygen consumption. There are several chemical substances that interrupt the mitochondrial electron transport chain (e.g., rotenone, antimycin A) and produce outcomes on tissue oxygenation comparable to that of cyanide. Oxygen extraction decreases in parallel with the decrease oxygen consumption, with an ensuing bigger venous oxygen content material and PvO2. Although cyanide stimulates the peripheral respiratory chemoreceptors, growing the stimulated oxygen fraction is no longer helpful, as there is already an ample quantity of oxygen which couldn't be used by the poisoned cells.

5. **Fulminating hypoxia**: It is caused via the inhalation of undiluted inert gases such as methane, nitrogen in lungs. Nitrous oxide can also cause fulminating hypoxia.
   Ischemic hypoxia: It was newly discovered caused due to flawed blood drift in tissue injuries. This may include a coronary heart assault that decreases normal blood flow or trauma to a tissue that outcome in damage. An instance of inadequate blood waft inflicting neighborhood hypoxia is gangrene that takes place in diabetes (Levin ME et.al., 1993)
   Ischemic hypoxia is somehow like stagnant hypoxia.

INTESTINE - ANATOMY

The intestines are a long, continuous tube running from the stomach to the anus, utmost absorption of nutrients and water occurs in the intestines. The intestines consist of the small intestine, large intestine and rectum.

Small Intestine

Chyme protrude from the belly enters the small intestine, and is the important digestive organ in the body. The longest phase of the alimentary canal, the small gut is about 3.05 meters lengthy in a dwelling person. Since this makes it about five instances longer than the large intestine, the identity of small intestine is derived from its incredibly smaller diameter of about 2.54 cm, in contrast with 7.62 cm for the large intestine.

Small intestine is subdivided into three regions.

- **Duodenum**
- **Jejunum**
- **Ileum**

The shortest place is the 25.4-cm duodenum, which starts at the pyloric sphincter. Just previous the pyloric sphincter, it bends posteriorly at the back of the peritoneum, turning into retroperitoneal, and then makes a C-shaped curve round the head of the pancreas earlier than ascending anteriorly once more to return to the peritoneal cavity and be a part of the jejunum. The duodenum can consequently be subdivided into 4 segments: the superior, descending, horizontal, and ascending duodenum.

Located in the duodenal wall, the ampulla marks the transition from the anterior component of the alimentary canal to the mid-region, and is the place the bile duct and the primary pancreatic duct join. This ampulla opens into the duodenum at a tiny volcano-shaped shape known as the foremost duodenal papilla. The hepatopancreatic sphincter (sphincter of Oddi) regulates the go with the flow of each bile and pancreatic juice from the ampulla into the duodenum.

The jejunum is about 0.9 meters lengthy and runs from the duodenum to the ileum. No clear demarcation exists between the jejunum and the ileum.

Ileum, measuring 1.8 meters is the longest phase of the small intestine. It is thicker, greater vascular, and has extra developed mucosal folds than the jejunum. It joins the cecum at the ileocecal sphincter. The jejunum and ileum are tethered to the posterior stomach wall by using the mesentery.

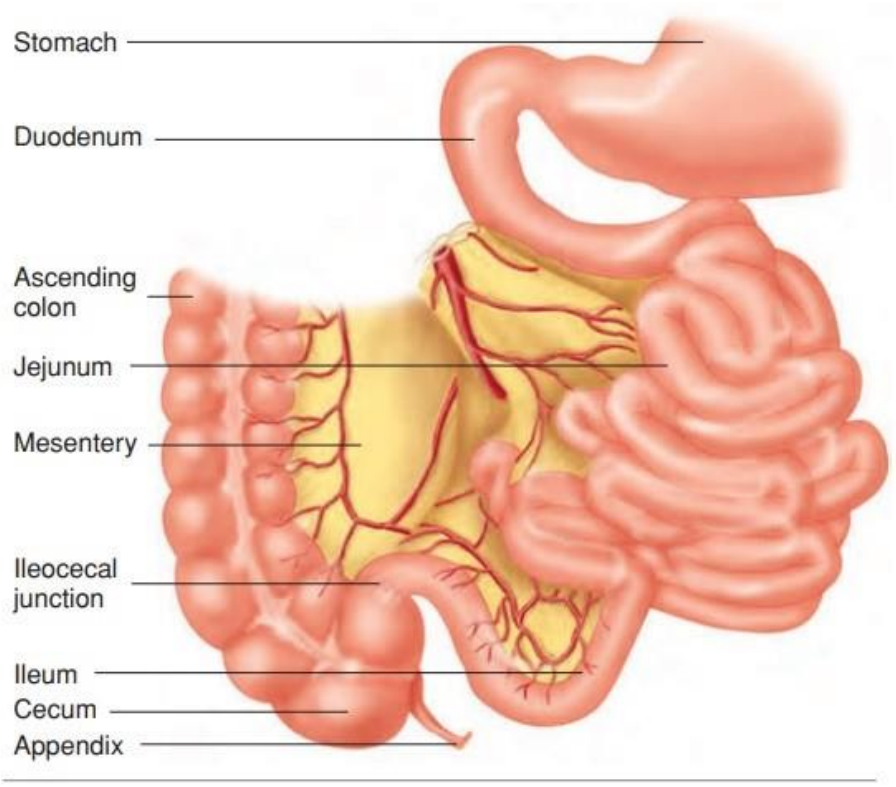

Fig.2- Small intestine (Cited from Textbook-Anatomy and Physiology)

The wall of the small gut is composed of the equal 4 layers mucosa, submucosa, muscularis externa and serosa usually existing in the alimentary system. However, three aspects of the mucosa and submucosa are unique. These features, which expand the absorptive floor vicinity of the small gut greater than 600-fold, encompass round folds, villi, and microvilli (Figure 2). These variations are most considerable in the proximal two-thirds of the small intestine, the place the majority of absorption occurs.

Large intestine

The large intestine is the end part of the alimentary canal. The essential characteristic of this organ is to end absorption of vitamins and water, synthesize vitamins, structure and remove feces from the body. The large intestine runs from the appendix to the anus. It covers the small intestine from three sides. It is subdivided into four main regions:

- Cecum
- Colon
- Rectum
- Anus

- **Cecum**

It is the first part of the large intestine and is a sac-like structure that is suspended inferior to the ileocecal valve. It is about 6 cm (2.4 in) long, receives the contents of the ileum, and continues the absorption of water and salts.

- **Colon**

The cecum blends into the colon. Reaching the colon, the meals residue first travels up the ascending colon. At the inferior of the liver, the colon bends to structure the proper colic flexure (hepatic flexure) and becomes the transverse colon. Food residue passing through the transverse colon travels throughout to the left facet of the abdomen. Further, meals residue passes via the descending colon, which flows down the left facet of the posterior belly wall. After coming into the pelvis inferiorly, it turns into the s-shaped sigmoid colon (Figure 3).

- **Rectum**

After sigmoid colon food residue enters the rectum in the pelvis. It is 20.3 cm (8 in) and extends anterior to the sacrum and coccyx. It has three lateral bends that create a trio of inner transverse folds recognized as the rectal valves.

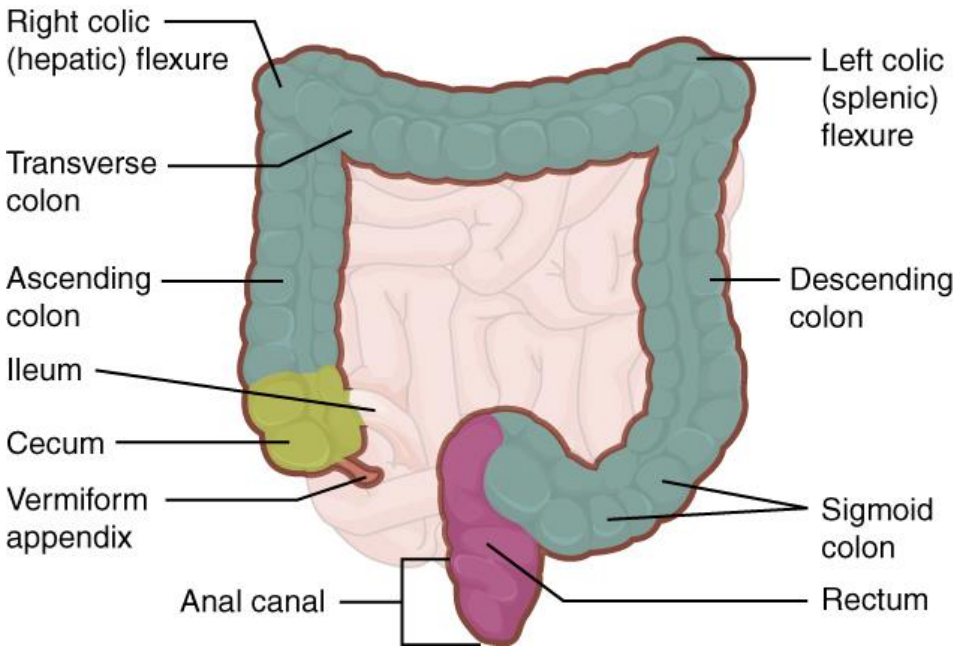

Fig. 3 -Large intestine (Cited from Textbook-Anatomy and Physiology)

- **Anal canal**

At last, meals residue reaches the final section of the large intestine, the anal canal, which is positioned in the perineum. It opens to the exterior of the body at the anus and consists of two sphincters. The interior anal sphincter is made of smooth muscle, and its contractions are involuntary while the exterior anal sphincter is made of skeletal muscle, which is underneath voluntary control.

Sprague Dawley Rat

The Sprague Dawley rat is an outbred multipurpose breed of albino rat used significantly in scientific and nutritional research. Its major gain is its calmness and ease of handling. This breed of rat was first produced by Robert S. Dawley in Sprague-Dawley farms in Madison, Wisconsin. The average litter size of the Sprague Dawley rat is 11.0.

Key characteristics:

- Most widely used outbred rat in biomedical research
- Excellent reproductive performance, making it an ideal model for generating timed-pregnant females
- Docile nature
- Coat Color: Albino

Fig.4- Experimental animal used **- Male Sprague Dawley Rat (SD Rat)** (cited from https://www.taconic.com/images/sprague-sawley-outbred-rat-model.jpg)

The experiments were conducted using male Sprague Dawley Rat ideally weighing between 180-200 grams. The male rats were used as they are more viable than female rats.

REVIEW OF LITERATURE

Each epithelium of intestine contains all these structures like brush border, villi, basolateral plasma membrane structure crypt. The small gut now not solely helps in absorbing vitamins from the food however additionally offers a bodily barrier and an organic barrier. The physical barrier that is tight junctions shaped via neighboring epithelial cells.The physical barrier that is tight junctions shaped by neighbouring epithelial cells and an organic barrier, each act in opposition to extra cellular materials such as microorganisms, antigens and xenobiotics (Figure 5). Furthermore, the small gut secretes an extensive vary of hormones that modify its inside features as properly as energy metabolism throughout the body.

The predominant characteristic of the small gut is the absorption of nutrients. The intestinal epithelium releases digestive enzymes that are transporters of specific nutrients, and metabolic enzymes. It additionally mediates sign transduction and produces bioactive compounds. The intestinal epithelium responds to a number of inflammatory and oxidative stresses brought on with the aid of proinflammatory cytokines (TNF-α and IL-1β), bacterial toxins and different elements thru a number of receptors, consisting of toll-like receptors (TLRs) which are existing on the plasma membrane of the epithelium. Cytokine receptors and intestinal cells expressing (TLRs) respond to lipopolysaccharides (LPS) and proinflammatory cytokines prompting intracellular signaling pathways.

The physical intestinal barrier is regulated by means of TJs via the paracellular motion of ions, solutes, and water throughout the intestinal epithelium and the detoxification device contributes to the organic barrier in opposition to xenobiotics. Moreover, TJ integrity is associated to the features of the intestinal epithelium (Lee B, et.al, 2018).

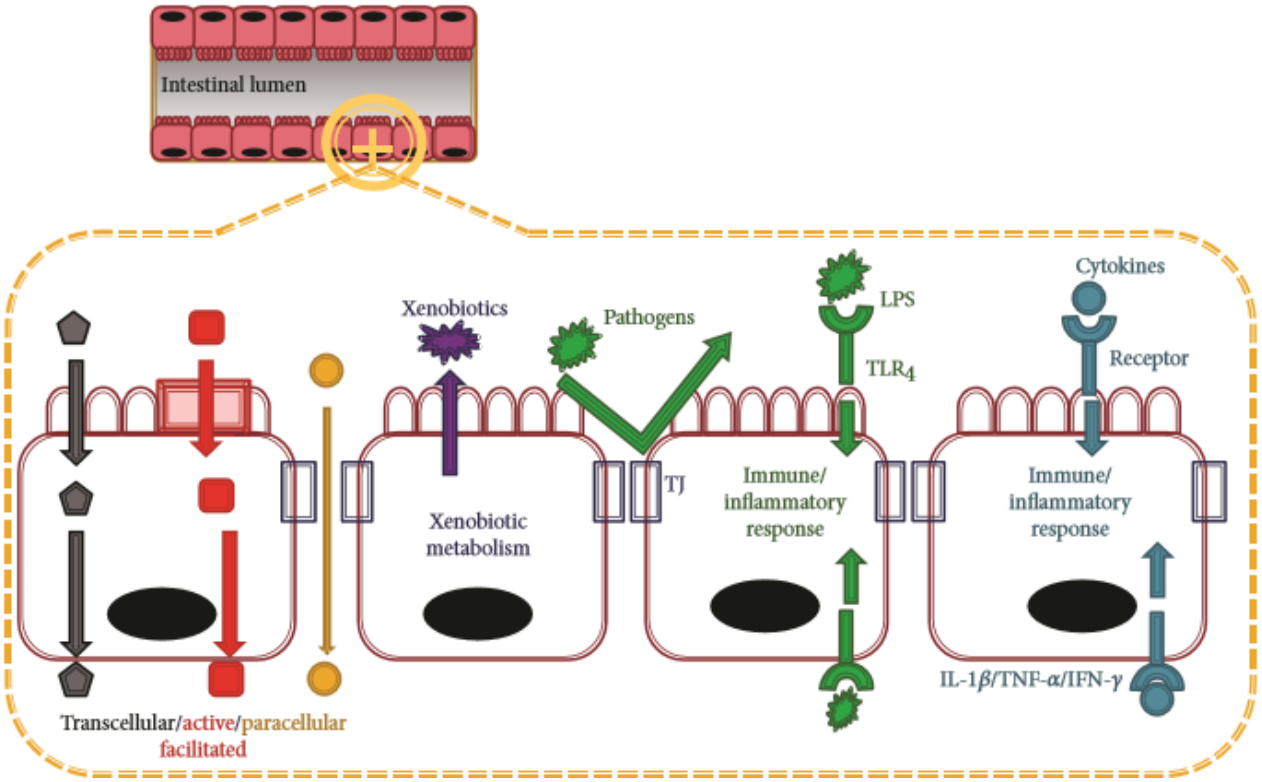

Fig. 5 - Functions of small intestine-performs barrier functions because of presence of tight junctions and xenobiotics detoxification system (Cited from Lee B, et. al., 2018)

Tight junctions

Tight Junctions assist to hold the bodily intestinal barrier via regulating the paracellular motion of ions, solutes, and water throughout the intestinal epithelium. TJ barrier performs an indispensable position in the pathogenesis of systemic and intestinal disorders.

TJ is generated via the meeting of more than one protein that are placed close to the apical section of the epithelium between neighboring cells (Figure 6) and controls the permeability of the paracellular transport pathway. It additionally performs a key function in keeping intestinal barrier function.

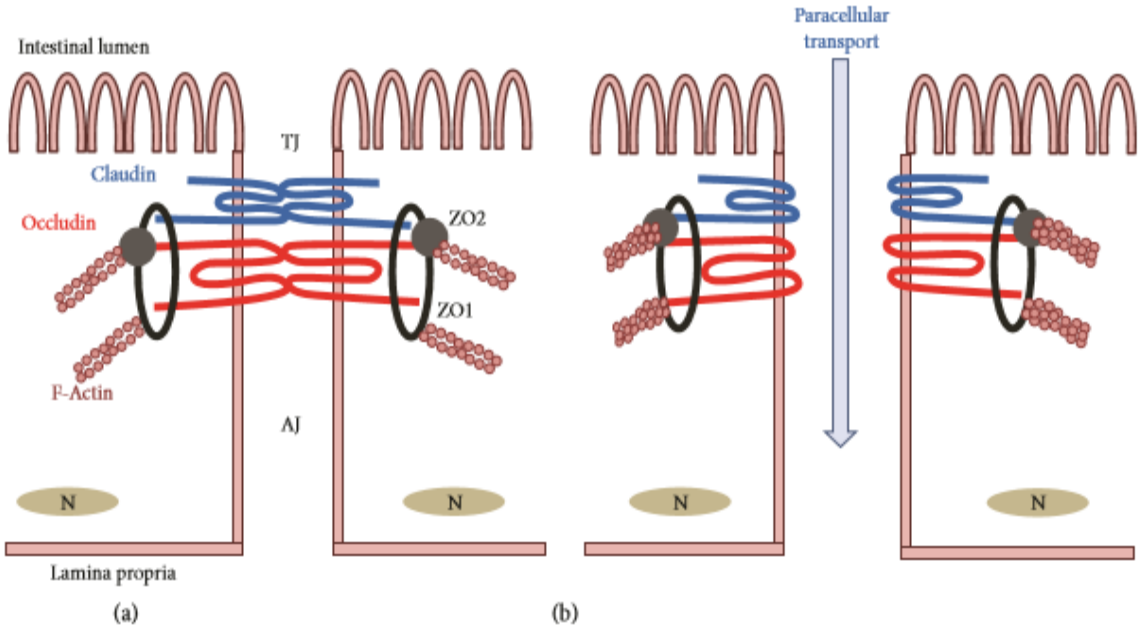

Fig. 6 (a) Meeting of TJ related proteins, (b) when luminal stimulation, the TJ opens, leading extracellular components to paracellular transport (Cited from Lee B, et. al., 2018).

Tight junctions comprise of two functional protein categories:

- Integral membrane proteins
- Peripheral membrane/Plaque proteins

Integral membrane protein

- Occluding: Occludin is the important protein which helps in maintenance of TJ and is expressed at cell-cell contact sites. It comprises of four transmembrane domains and two extracellular loops. Less phosphorylated occludin is found in the cytoplasm while the phosphorylated occludin at its serine/ threonine residue is localized mainly in the membrane.
- Phosphorylation of occludin seems to control its interaction with other TJ proteins such as ZO-1(Rao R, et al., 2009). For TJ integrity, the interplay between occludin and ZO-1 is crucial (Bazzoni G, et al., 2004). Therefore, phosphorylated occludin regulates TJ stability and permeability. A greater degree of occludin performs a function in addition enhancing TJ barrier characteristic and stopping injury to the TJ.

- Claudin: Four transmembrane domains and two extracellular loops that assemble TJ strands are found in claudin (Figure 5). It's family is composed of 23 integral membrane proteins. The extracellular loops of claudin take part in heterophilic and hemophilic interactions with adjoining cells, with the help of which boundaries are generated in opposition to pores for allowing the movement of particular molecules in the paracellular pathways (Van Itallie, et al., 2006).

Peripheral membrane proteins

- Zonula Occludens. The ZO protein is the first TJ-specific protein to be discovered (Haskins J, et al., 1998). It is of three kinds that is ZO-1, ZO-2 and ZO-3. Junctional proteins such as occludin, claudin and the actin cytoskeleton are joined by ZO, and the resulting protein interactions keep TJ formation and functioning.
  Studies point out that though ZO-1-deficient cells can preserve the shape of TJs and showcase regular permeability, the endeavor of different TJ proteins such as occludin and claudins in assembling TJs used to be delayed in these cells (Umeda K et al., 2004). On the different hand, the lack of ZO-2 or ZO-3 did not affect the formation of TJ in epithelial cell types, which suggests that ZO-1 proteins play a more crucial role in the management of TJ assembly in contrast to ZO-2 or ZO-3.

Effect of Hypoxia on tight junction proteins

Tight junctions form the spine for the shape of the epithelial barrier and additionally represent to the bodily groundwork for a permeability barrier to solutes and ions (Capaldo CT, et al., 2015). The TJ includes both transmembrane and peripheral membrane proteins tightly linked to the actin-based cytoskeleton (Ivanov AI et al., 2010). Hurdle-function occurs as a result of TJs additionally stopping lipid diffusion between apical and basolateral membrane domains (Bacalao R, et al., 1994).TJ complicated meeting and transcriptional manage of its elements are tightly regulated through a range of physiological and pathophysiological stimuli (Koch S, et. al., 2012).

Hypoxia influences the integrity of tight junction, ensuing in loss of barrier feature and transepithelial electrical resistance. This has been located the use of chemical depletion of ATP depletion (Tsukamoto T, et al., 1999) and in vitro hypoxia (Taylor CT, et al., 1998). The permeability modifications are additionally imputable to ameliorations in the distribution of occludin, zonula occludens-1 (ZO-1), ZO-2, and cingulin. TJ integrity is additionally influenced via perturbations of the interplay with the actin-based cytoskeleton and by using the degradation of membrane cytoskeletal proteins such as ankyrin and fodrin in ATP depletion models.

Claudins are a massive household of tetra spanning necessary membrane proteins that are uniquely assist in presenting the selective permeability to TJs (Capaldo CT, et al., 2015). They are categorized as tight or leaky in contrast to their impact on barrier function. Claudin-1 (CLDN1) that is a necessary tight claudin has been proved to be dysregulated in several human diseases, including inflammatory bowel disease (IBD). In a latest display screen of TJ targets, CLDN1 used to be recognized as a central mediator of aberrant junctional morphology in HIF1β–deficient intestinal epithelial cell (IEC) traces (Saeedi BJ, et al., 2015). CLDN1 expression is continued by HIF through binding hypoxia response

element (HRE) sequences in the gene promoter. Barrier characteristic and morphologic abnormalities are maintained by the reintroduction of CLDN1 into HIF-1β–deficient cells. Moreover, in vivo evaluation printed the significance of HIF-mediated CLDN1 expression at some point of experimental colitis. These effects discover an essential hyperlink between HIF and precise TJ function, offering necessary perception into mechanisms of HIF-regulated epithelial homeostasis (Saeedi BJ, et al., 2015).

Quercetin

The most extensively disbursed flavonoids in plants. Fruits, vegetables, and grains, crimson onions and kale are frequent ingredients containing considerable quantities of quercetin. It has a bitter taste and is used as an ingredient in dietary supplements, beverages, and foods. It is a flavonoid broadly dispensed in nature (Flavonoids (Review), 2015). The title has been used when you consider that 1857, and is derived from quercetum (oak forest), after Quercus. It is a naturally occurring polar auxin transport inhibitor (Fischer C, et al., 1997). The International Union of Pure and Applied Chemistry (IUPAC) nomenclature for quercetin is 3, 31, 41, 5, 7-pentahydroxyflvanone. This skill that quercetin has an OH crew connected at positions 3, 5, 7, 31, and 41.

In red onions, greater concentrations of quercetin take place in the outermost rings and in the section closest to the root, the latter being the section of the plant with the best awareness (Slimestad R, et al., 2007). One learns about determined that organically grown tomatoes had 79% greater quercetin than non-organically grown fruit(Mitchell et al.,2007).Quercetin is current in a variety of types of honey from distinctive plant sources (Petrus et al., 2011).

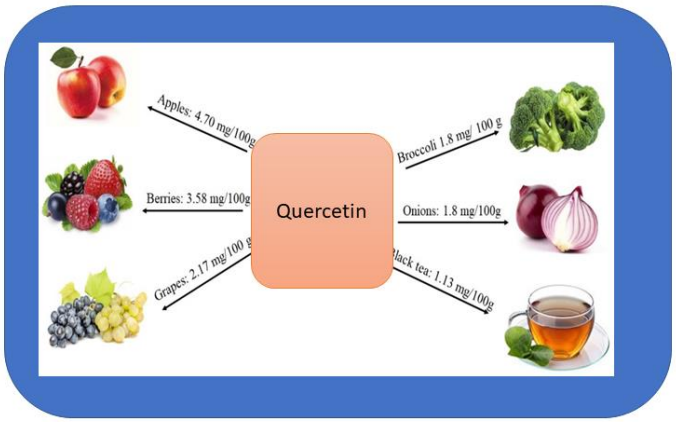

Fig. 7- Ray diagram of natural sources of quercetin

Quercetin (C15H10O7) is an aglycone, missing a connected sugar. It is superb citron yellow needle crystal and totally insoluble in bloodless water, poorly soluble in warm water however pretty soluble in alcohol and lipids. It has special organic homes that might also enhance mental/physical overall performance and limit contamination threat (Davis JM et al., 2009). These residences shape the groundwork for viable benefits to ordinary fitness and disorder resistance, together with anti-carcinogenic, anti-inflammatory, antiviral, antioxidant, and psycho stimulant activities, as properly as the capacity to inhibit lipid peroxidation, platelet aggregation and capillary permeability, and to stimulate mitochondrial biogenesis (Aguirre L et al., 2011).

Quercetin and Tight junctions

TJ is related with physical intestinal barrier function and regulates the paracellular motion of more than a few supplies throughout the intestinal epithelium. Studies expose that TJ dysfunction is intently associated to inflammatory and metabolic problems which include IBD, NASH, NAFLD, and weight problems through the disruption of TJ barrier functions. Thus, the protection of TJ integrity is possibly a true approach to forestall and/or deal with these diseases. Of its many organic activities, Quercetin is established for defending cells from oxidative and inflammation-associated injuries. The organic features of quercetin are intently related with the legislation of key enzymes inclusive of PI3kinase, NF-κB, PKC, tyrosine kinase, and the MAPK household (Suzuki T et al., 2011; Ulluwishewa D et al., 2011). Enzymes (especially PKC and MAPKs) and their downstream signaling pathways are carefully associated to the meeting and integrity of TJ features (Suzuki T et al., 2011). Thus, quite a number research has been underneath taken to elucidate the roles of quercetin in TJ integrity.

Quercetin augmented TJ barrier characteristic in Caco-2 cells in the absence of any stimuli such as proinflammatory cytokines (Amasheh M, et al., 2008). Expression of claudin-4 however now not different TJ proteins such as occludin and claudin-1, -3, and -7 had been multiplied by way of therapy with 200μM quercetin for 24 hours. Another finds out about confirmed that quercetin cure extended the transepithelial electrical resistance (TER) throughout the monolayers and decreased lucifer yellow flux, a paracellular marker (Suzuki T, et. al., 2009).

In order to become aware of the mobile mechanisms concerned in the beneficial effect of quercetin on TJ, quite a few protein kinase inhibitors had been used. H7, an inhibitor of PKA and PKG and Staurosporine, an established protein kinase inhibitor, abrogated the preventive characteristic of quercetin on TJ, indicating that the inhibition of PKA and PKG contributes to the shielding effect on TJ by quercetin. Another findings said that 100μM quercetin reinforces TJ integrity thru the modulation of a couple of TJ-related proteins together with claudin-1 and -4, ZO-2, and occludin by way of suppressing PKCδ (Suzuki T, et al., 2009). Thus, it seems that the suppression of a couple of protein kinases contributes to quercetin-mediated TJ integrity.

Quercetin -Inflammation and immune function

Quercetin is mentioned to act as a long lasting anti-inflammatory substance that possesses robust anti-inflammatory capacities (Read MA, et al., 1995; Oršolić N, et al., 2004). It possesses anti-inflammatory cells that can be expressed on each in animal and human (Ghosh, et al., 1999). Quercetin inhibits manufacturing of inflammation-producing enzymes (cyclooxygenase (COX) and lipoxygenase (LOX)) (Kim HP, et al., 1998). It is recognized to possess each mast cell stabilizing and gastrointestinal

cytoprotective activity. It can additionally play a modulating, biphasic and regulatory motion on inflammation and immunity. Moreover, quercetin has an immune suppressive impact on dendritic cells feature (Huang RY, et al., 2010).

Quercetin impacts immunity and inflammation with the aid of appearing usually on leukocytes and concentrated on many intracellular signaling kinases and phosphatases, enzymes and membrane proteins are frequently vital for a cell specific feature (Li Y, et al., 2016). Quercetin most probable universally suppresses the accumulation and activation of immune cells, which includes anti-inflammatory cells, whereas it specifically will increase gene expression related with mitochondrial Oxidative Phosphorylation.

Inflammatory Bowel Disease

Hypoxia exhibits a role in IBD. Lower resting oxygen levels have been demonstrated in sections of IBD tissue compared to the controls using a 2-nitroimidazole based approach (Karhausen J et. al., 2004).

IBD includes an extensive vary of continual revoking illnesses of which crohn's disease and ulcerative colitis (CD and UC respectively) are in all likelihood found mostly (Holmberg FE et al., 2018). IBD, regarded to contain an excessive stage of intestinal inflammation, is additionally related with dysregulation of TJ (Edelblum KL et al., 2009).IBD affect many aspects of the epithelial barrier which consists of changes in the epithelium, its adhesion molecules and the altered manufacturing of antimicrobial peptides and mucus. Altogether, these changes end result in the loss of solutes and fluid at some stage in the epithelial barrier, main to leak-flux diarrhea and exalted antigen translocation (Holmberg FE et al., 2018). Antigen translocation in the lamina propria reasons inflammation derived from circulating and resident immune cells, main to similarly disruption of the barrier characteristic (Holmberg FE et al., 2018; McGuckin MA et al., 2009).

The intestinal mucosa is uncovered to steep hypoxic gradients (Karhausen J et al., 2004) and is in a steady nation of managed inflammation, which is imperative to enable tolerance to in any other case innocent ingested dietary antigens (Fig. 10) (Poonam P, 2007). This stability is pathologically disturbed in inflammatory bowel disease (IBD); a relapsing-remitting revolutionary disease of the gastrointestinal tract that consists of each Crohn's and ulcerative colitis. The signs and symptoms of IBD can vary from moderate to extreme and encompass stomach pain, intestinal bleeding, weight loss, fever and diarrhea (Podolsky DK, 1991). The two IBD sub-types have special distribution patterns: ulcerative colitis is confined to the colon, whereas Crohn's colitis can have an effect on any phase of the GI tract. Both are idea to show up when inappropriate immunological endeavor in the intestinal mucosa outcomes in epithelial barrier dysfunction main to publicity of the mucosal immune gadget to luminal antigenic cloth and similarly cycles of irritation and barrier dysfunction that underlie disorder development (Cummins EP et al., 2013: Abraham C, et al., 2009).

Redistribution and reduced expression of claudin-1, claudin-4, claudin-7, and occludin, as properly as a markable extend in claudin-2 expression is a reflection of active UC. CD on the different hand is additionally related with each the redistribution and lowered expression of claudin-3, claudin-5, and claudin-8, as nicely as accelerated expression of claudin-2 (Hering NA et al., 2012; Luettig J et al., 2015). Furthermore, CD is discovered to current a peculiar intestinal shape with an excessive degree

of intestinal inflammation (Hollander D et al., 1998). For example, sufferers with CD have increased degrees of plasma, fecal, and intestinal TNF-αs, which additionally can expediate TJ dysfunction (Gibson PR, 2015). Altogether, the redistribution and alteration of TJ proteins, as properly as inflammatory responses, are intently related with barrier dysfunction in sufferers with UC or CD.

In the context of IBD, HIF system activity is idea to be protective, performing thru three mechanisms: i) inhibition of epithelial expression of barrier-protective genes; and the iii) apoptosis; ii) stronger merchandising of neutrophil apoptosis (Fig. 8) (Cummins EP et al., 2013). Evidence of the anti-apoptotic results of HIF has been tested circuitously thru experiments to look into the position of the hydroxylase inhibitor, dimethyloxalylglycine (DMOG), in colitis.

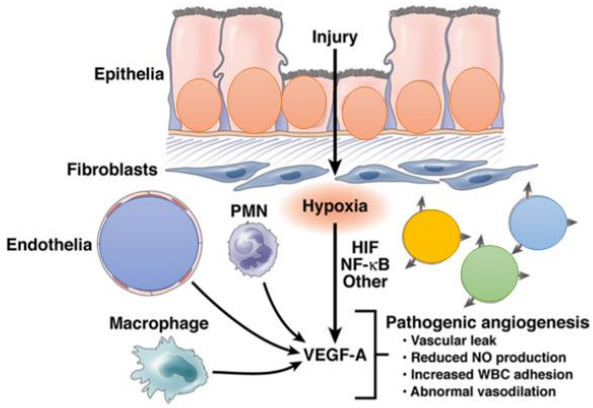

Fig. 8-The ray diagram showing effect of hypoxia on inflammatory bowel disease

In addition to its anti-apoptotic effects, the HIF machine can defend in opposition to colitis via the expression of barrier shielding genes. Several HIF-dependent goal genes have been proposed as mediators of this effect: CD55 (Louis NA et al., 2005), ecto-50 nucleotidase (Synnestvedt K et al., 2002), A2B receptor (Kong T et al., 2006) MUC-3 (Louis Na et al., 2006), intestinal trefoil issue (Furuta GT et al., 2001), and P-glycoprotein (Comerford KM, et al., 2002) all play a function in the legislation of the intestinal mucosa barrier and have all been validated to be regulated in a hypoxia-dependent manner.

There is additional proof of the differential outcomes of the HIF-α isoform in IBD. HIF-2α expression has been proven to be accelerated in colon tissues of mice after the induction of colitis. This was once additionally found in sufferers with ulcerative colitis or Crohn's ailment (Xue X et al., 2013). Interestingly, in that study, whilst the loss of HIF-2α used to be related with attenuated colonic inflammation, the over expression of HIF-2α led to spontaneous colitis and expanded inflammation.

Oxidative stress

When a small amount of consumed oxygen is decreased in a precise way the breathing cells yield to a range of incredibly reacting chemical entities, at the same time recognized as reactive oxygen species (ROS). These ROS are successful of inflicting injury to the macromolecules like lipid peroxidation; oxidation of amino acid facet chains (especially cysteine); formation of protein-protein move links; V oxidation of polypeptide spine ensuing in protein fragmentation and DNA strand breaks etc. Small quantity of ROS produced as an end result of electron switch response in mitochondria, peroxisomes and cytosol are scavenged through mobile defending structures which includes enzymatic and non-enzymatic antioxidants. A kingdom of somewhat accelerated degree of intracellular ROS is referred to as oxidative stress which produces free radicals viz., awesome oxide, hydrogen peroxide radicals and hydroxyl radicals

Free radicals are reactive molecular species with unpaired electrons that oxidize different molecules to achieve electrons and stabilize themselves. The response produces different free radicals, initiating a domino impact of free radical stabilization and formation (Machlin LJ et. al., 1987). Free radical injury can reason unsaturated bonds in membrane lipids to free fluidity when per oxidized and proteins to denature. The oxidative stress is carefully associated to inflammation, a pathologic technique characterized via activation of sure transcription factors.

The oxidative stress is intently associated to inflammation, a pathological system characterized by means of activation of the transcription thing NFkB (Nuclear factor kappa beta) (Døhlen G et. al., 2005).Normally NFkB exists in cytoplasm in an inactive shape related with regulatory proteins referred to as inhibitors of kB (IkB).The range of stimuli that spark off NFkB motive phosphorylation of IκB, which is accompanied by using its ubiquitination and subsequent degradation. This effect in publicity if the Nuclear Localization Signals (NLS) and the subsequent translocation of the molecules to the nucleus. In the nucleus NF-κB binds with the consensus sequence of a variety of genes and accordingly prompt their transcription (Jobin C et al., 1999).

OBJECTIVES

- Identify the effect of hypobaric hypoxia on tight junction proteins in Intestine of rat (Male Sprague Dawley).

- To identify changes in biochemical parameters –
    - Reactive oxygen species (ROS)
    - Malondialdehyde(MDA)
    - Glutathione (GSH)

MATERIALS AND METHODS

The present study involving the study of effect of hypoxia on tight junction proteins in intestine in rats was carried out under two main objectives:

1) To determine the effect of hypobaric hypoxia on tight junction proteins in intestine.

2) To identify changes in biochemical parameters.

Animals

Male Sprague Dawley (SD) rats were received from the central animal facility of DIPAS-DRDO, Delhi, India. Animals weighing between 180-200g were housed in experimentally designed polypropylene cages of 32in. ×24in. ×16in. dimension under standard conditions (25±2˚C temperature, 55±5% relative humidity and 12h light/dark cycle) and free retrieval to standard laboratory food and water ad libitum.

Full protocol including animal studies were evaluated and concurred by the Institutional Animal Ethics Committee (IAEC), DIPAS, Delhi, India, accredited to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. We have followed the standards set forth in the guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC).

Drug Administration (Quercetin dose)

The drug Quercetin (50mg/kg) BW was freshly dissolved in DMSO (0.5%) Dimethylsulphoxide individually and administrated orally to rats, 1hr prior to hypoxic exposure for 6 hours.

Experiment set up

The experiment was carried out as follows:

The rats were divided into 4 groups each group containing 6 rats each:

- Group 1 : Normoxia (Nor) – served as control
- Group 2 : Hypoxia (Hyp) – was exposed to hypoxia for 6 hours and no drug was administrated
- Group 3 : Normoxia+Quercetin (Nor+que) – was supplemented with quercetin at the rate of 50mg/kg BW
- Group 4 : Hypoxia+Quercetin (Hyp+que) – was supplemented with quercetin at the rate of 50mg/kg BW and exposed to hypoxia for 6 hours

Exposure to hypoxia

Simulated hypobaric hypoxia chamber (Matrix, India) was used to produce hypoxic conditions. Animals were kept in the chamber for 6h at an altitude of 7,620m (280mm Hg) with sustained temperature of 25±2˚C. This 6h of hypoxia exposure has been opted based on previous studies by our lab demonstrating the elevated transvascular leakage at 6h of hypoxia exposure time at 7620 m. Fresh air was flushed at the rate of 4l/h along with the relative humidity of 55±5% inside the hypoxia chamber. Moreover, the partial pressure of oxygen (PO2) in control rats was observed to be 95±2 mm Hg whereas, in hypoxic rat PO2 was 36±2mm Hg, indicating that the rats were exposed to low barometric pressure at high altitude. The animals were provided with food and water ad libitum during hypoxia exposure. Utmost care was taken to minimize animal sufferings while performing the experiments.

Biochemical parameters

Method of sacrifice: After 6h of hypoxia exposure, rats were sacrificed using Ketamine hydrochloride (80mg/kg BW) and Xylazine (20 mg/kg BW) as an anesthesia.
Ketamine contributes to hallucinations and a feeling of relaxation while xylazine functions as the muscle reluctant to the animal.

Sample preparation: Normoxia and hypoxia exposed animal lungs were perfused with cold 1X PBS followed by washing with saline (0.9% NaCl) and after that homogenized (10%) by 0.154 M KCL containing PMSF, DTT and protease inhibitor cocktail (PIC) for further processing of biochemical estimations.

Biochemical Parameters

After exposure, animals were sacrificed and blood was removed by perfusion with cold PBS. The intestine tissue was collected, washed with cold saline (0.9% NaCl) and further biochemical analysis was performed. The following biochemical parameters were determined.

1) Malondialdehyde (MDA) in intestine was estimated.

2) Estimation of Reactive Oxygen Species (ROS)-After hypoxic exposure, animals were sacrificed and intestine was perfused with cold PBS. The kidney tissue was collected washed with cold saline (0.9%NaCl) and stored at -80°C for further biochemical analysis. Reactive Oxygen Species (ROS) in intestine homogenate was estimated.

3) Reduced Glutathione (GSH) in intestine was determined.

RESULTS AND CONCLUSION

Changes in Biochemical Parameters

Reactive oxygen species (ROS):

Significant increase in ROS levels ($P<0.05$) in intestine tissue of animals exposed to hypoxia was found. Supplementation of quercetin under normoxia did not alter the ROS levels in animals. However, the same dose under hypoxia showed a significant reduction in ROS levels ($P<0.05$) in intestine homogenate of rats compared to hypoxia (6 h) (Figure 9).

Malondialdehyde (MDA)

An end product of lipid peroxidation that is MDA was found to be increased in the intestine of rats exposed to 6 hours of hypoxia. Animals supplemented with 50mg/kg BW of quercetin under normoxia did not alter the MDA levels in intestine tissues. However the levels of MDA was found to be reduced significantly under hypoxia with the administration of quercetin compared to the control (6 hours hypoxia without drugs) (Figure 10)

Antioxidant levels (GSH): Since there was an increased oxidative stress in hypoxia exposed animals, we determined the levels of antioxidant status, reduced glutathione (GSH) in intestine of these animals**.** The reduced glutathione (GSH--γ-L-glutamyl- L-cysteinyl- glycine) levels in the intestine tissues of animals exposed to hypoxia were reduced significantly ($P<0.05$) compared to control. However, quercetin supplementation during hypoxia increased GSH levels more or less similar to that of normoxia values (Figure 11)

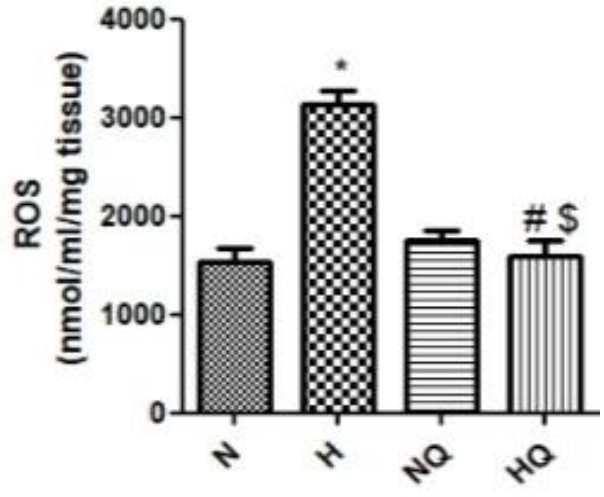

Figure 9- Reactive Oxygen Species (ROS) in kidneys of rats subjected to 50mg quercetin/kg BW, exposed to Hypoxia at 7260m, 25±1℃ for 6 hours.

All values are mean ± SD (n=6)

P<0.05

*=Normoxia vs Hypoxia

#=Hypoxia vs Hypoxia + Quercetin

N-Normoxia

H-Hypoxia (6h)

NQ-Normoxia + Quercetin (50mg/kg BW)

HQ-Hypoxia + Quercetin (50mg/kg BW)

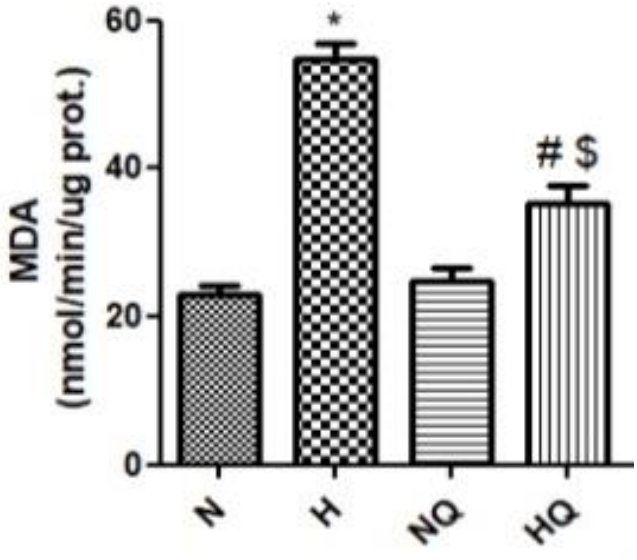

Figure: 10- Malondialdehyde (MDA) in intestine of rats subjected to 50mg quercetin/Kg of BW, exposed to hypoxia at 7260m, 25±1℃ for 6 hours.

All values are mean±SD (n=6)

P<0.05

*= Normoxia vs Hypoxia

=Hypoxia vs Hypoxia + Quercetin

N-Normoxia

H-Hypoxia (6h)

NQ-Normoxia+Quercetin (50mg/kg BW)

HQ-Hypoxia+Quercetin (50mg/kg BW)

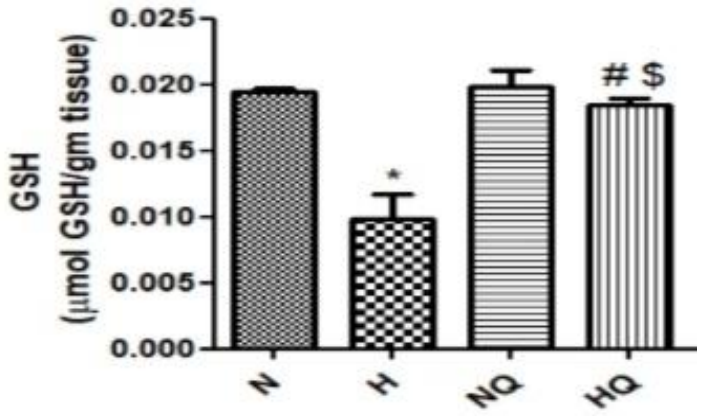

Figure: 11-Reduced glutathione (GSH) in kidneys of rats subjected to 50mg quercetin/kg BW, exposed to Hypoxia at 7260m, 25±1℃ for 6 hours,

All values are mean± SD (n=4)

P<0.05

*=Normoxia vs Hypoxia

#=Hypoxia vs Hypoxia + Quercetin

N-Normoxia

H-Hypoxia (6h)

NQ-Normoxia+Quercetin (50mg/kg BW)

HQ-Hypoxia+Quercetin (50mg/kg BW)

DISCUSSION

The ability to sense and respond to the changes in the oxygen is essential for the survival of prokaryotic and eukaryotic organisms. Oxygen-sensing mechanisms have been developed to maintain cell and tissue homeostasis. Oxygen is necessary for growth of life and proper oxygen is required for respiration also.

Under hypoxic conditions, the cells are more susceptible to oxidative stress which is closely related to inflammation. During inflammation, the transcription factors like Hif-1α get upregulated. Hypoxia paradoxically stimulates ROS released from the mitochondria that subsequently regulate the transcriptional and post transcriptional response to low oxygen conditions. In the present study, we observed that, reactive oxygen species levels (ROS) and MDA levels were significantly increased under hypoxia. Pretreatment with quercetin significantly reduced the ROS and MDA levels in intestine of rats under hypoxia. The antioxidant GSH levels were found to be decreased under hypoxia compared to control while prior treatment with quercetin reversed these changes under hypoxia by increasing the GSH levels significantly compared to control animals. The same dose of Quercetin (50mg/kg BW) supplementation under normoxia showed unmodified GSH levels, indicating that during the stress (Hypoxia), GSH synthesis is increased to cope up with the oxidative stress which is an adaptive phenomenon. This is attributed to the fact that quercetin can modulate the key cell signaling pathways to robustly enhance the synthesis of antioxidant glutathione.

The natural anti-inflammatory products are beneficial for inflammatory disorders. Quercetin stimulates antioxidant enzymes. Quercetin is considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions. These properties of quercetin allow it to inhibit lipid peroxidation.

REFERENCES

Aguirre L, Arias N, Teresa Macarulla M, Gracia A, P Portillo M. Beneficial effects of quercetin on obesity and diabetes. The Open Nutraceuticals Journal. 2011 Nov 3;4(1).

Amasheh M, Schlichter S, Amasheh S, Mankertz J, Zeitz M, Fromm M, Schulzke JD. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. The Journal of nutrition. 2008 Jun 1;138(6):1067-73.

Abraham C, Cho JH. Mechanisms of disease. N Engl J Med. 2009;361:2066-78.

Biddlestone J, Bandarra D, Rocha S. The role of hypoxia in inflammatory disease. International journal of molecular medicine. 2015 Apr 1;35(4):859-69.

Bacallao R, Garfinkel A, Monke S, Zampighi G, Mandel LJ. ATP depletion: a novel method to study junctional properties in epithelial tissues. I. Rearrangement of the actin cytoskeleton. Journal of Cell Science. 1994;107(12):3301-13.

Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiological reviews. 2004 Jul;84(3):869-901.

Cummins EP, Doherty GA, Taylor CT. Hydroxylases as therapeutic targets in inflammatory bowel disease. Laboratory investigation. 2013 Apr;93(4):378-83.

Capaldo CT, Nusrat A. Claudin switching: physiological plasticity of the tight junction. Semin Cell Dev Biol. 2015;42:22–29

Cafaro RP. Hypoxia: Its causes and symptoms. Journal of the American Dental Society of Anesthesiology. 1960 Apr;7(4):4.

Cummins EP, Doherty GA, Taylor CT. Hydroxylases as therapeutic targets in inflammatory bowel disease. Laboratory investigation. 2013 Apr;93(4):378-83.

Colledge NR, Walker BR, Ralston SH, editors. Davidson's principles and practice of medicine. Edinburgh; New York: Churchill Livingstone/Elsevier; 2010 Mar 22.

Cymerman A, Rock PB. Medical Problems in High Mountain Environments. A Handbook for Medical Officers. ARMY RESEARCH INST OF ENVIRONMENTAL MEDICINE NATICK MA THERMAL AND MOUNTAIN MEDICINE DIVISION; 1994.

Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer research. 2002 Jun 15;62(12):3387-94.

Davis JM, Murphy EA, Carmichael MD. Effects of the dietary flavonoid quercetin upon performance and health. Current sports medicine reports. 2009 Jul 1;8(4):206-13.

Døhlen G, Carlsen H, Blomhoff R, Thaulow E, Saugstad OD. Reoxygenation of hypoxic mice with 100% oxygen induces brain nuclear factor-kappa B. Pediatric research. 2005 Nov;58(5):941-5.

Das KK, Honnutagi R, Mullur L, Reddy RC, Das S, Majid DS, Biradar MS. Heavy metals and low-oxygen microenvironment—its impact on liver metabolism and dietary supplementation. In Dietary Interventions in Liver Disease 2019 Jan 1 (pp. 315-332). Academic Press.

Edelblum KL, Turner JR. The tight junction in inflammatory disease: communication breakdown. Current opinion in pharmacology. 2009 Dec 1;9(6):715-20.

Flavonoids (Review)". Micronutrient Information Center, Linus Pauling Institute, Oregon State University, Corvallis, OR. November 2015. Retrieved 1 April 2018.

Fischer C, Speth V, Fleig-Eberenz S, Neuhaus G. Induction of zygotic polyembryos in wheat: influence of auxin polar transport. The Plant Cell. 1997 Oct 1;9(10):1767-80.

Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, Podolsky DK, Colgan SP. Hypoxia-inducible factor 1–dependent induction of intestinal trefoil factor protects barrier function during hypoxia. The Journal of experimental medicine. 2001 May 7;193(9):1027-34.

Glover LE, Colgan SP. Epithelial Barrier Regulation by Hypoxia-Inducible Factor. Annals of the American Thoracic Society. 2017 Sep;14(Supplement 3):S233-6.

Gibson PR. Increased gut permeability in Crohn's disease: is TNF the link?. Gut. 2004 Dec 1;53(12):1724-5.

Glover LE, Lee JS, Colgan SP. Oxygen metabolism and barrier regulation in the intestinal mucosa. The Journal of clinical investigation. 2016 Oct 3;126(10):3680-8.

Ghosh B. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-α production in murine macrophages. International journal of immunopharmacology. 1999 Jul 1;21(7):435-43.

Huang RY, Yu YL, Cheng WC, OuYang CN, Fu E, Chu CL. Immunosuppressive effect of quercetin on dendritic cell activation and function. The Journal of Immunology. 2010 Jun 15;184(12):6815-21.

Hillman K, Bishop G. Clinical intensive care and acute medicine. Cambridge University Press; 2004 Mar 11.

Haskins J, Gu L, Wittchen ES, Hibbard J, Stevenson BR. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. The Journal of cell biology. 1998 Apr 6;141(1):199-208.

Hering NA, Fromm M, Schulzke JD. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. The Journal of physiology. 2012 Mar 1;590(5):1035-44.

Holmberg FE, Pedersen J, Jørgensen P, Soendergaard C, Jensen KB, Nielsen OH. Intestinal barrier integrity and inflammatory bowel disease: Stem cell-based approaches to regenerate the barrier. Journal of Tissue Engineering and Regenerative Medicine. 2018 Apr;12(4):923-35.

Hollander D. Crohn's disease--a permeability disorder of the tight junction?. Gut. 1988 Dec;29(12):1621.

Ivanov AI, Parkos CA, Nusrat A. Cytoskeletal regulation of epithelial barrier function during inflammation. The American journal of pathology. 2010 Aug 1;177(2):512-24.

Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Sartor RB. Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity. The Journal of Immunology. 1999 Sep 15;163(6):3474-83

Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. The Journal of clinical investigation. 2004 Oct 15;114(8):1098-106.

Kim HP, Mani I, Iversen L, Ziboh VA. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. Prostaglandins, leukotrienes and essential fatty acids. 1998 Jan 1;58(1):17-24.

Koch S, Nusrat A. The life and death of epithelia during inflammation: lessons learned from the gut. Annual Review of Pathology: Mechanisms of Disease. 2012 Feb 28;7:35-60.

Kong T, Westerman KA, Faigle M, Eltzschig HK, Colgan SP. HIF-dependent induction of adenosine A2B receptor in hypoxia. The FASEB Journal. 2006 Nov;20(13):2242-50.

Louis NA, Hamilton KE, Canny G, Shekels LL, Ho SB, Colgan SP. Selective induction of mucin-3 by hypoxia in intestinal epithelia. Journal of cellular biochemistry. 2006 Dec 15;99(6):1616-27.

Li Y, Yao J, Han C, Yang J, Chaudhry MT, Wang S, Liu H, Yin Y. Quercetin, inflammation and immunity. Nutrients. 2016 Mar;8(3):167.

Levitan, Richard (2020-04-20). *"Opinion | The Infection That's Silently Killing Coronavirus Patients"*. The New York Times. *ISSN 0362-4331*. *Retrieved 2020-04-22*.

Levin ME, O'Neal LW, Bowker JH. The diabetic foot. Mosby; 1993 Jan 15.

Lee B, Moon KM, Kim CY. Tight junction in the intestinal epithelium: Its association with diseases and regulation by phytochemicals. Journal of immunology research. 2018;2018.

Luettig J, Rosenthal R, Barmeyer C, Schulzke JD. Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. Tissue barriers. 2015 Apr 3;3(1-2):e977176.

Louis NA, Hamilton KE, Kong T, Colgan SP. HIF-dependent induction of apical CD55 coordinates epithelial clearance of neutrophils. The FASEB Journal. 2005 Jun;19(8):950-9.

Mitchell AE, Hong YJ, Koh E, Barrett DM, Bryant DE, Denison RF, Kaffka S. Ten-year comparison of the influence of organic and conventional crop management practices on the content of flavonoids in tomatoes. Journal of agricultural and food chemistry. 2007 Jul 25;55(15):6154-9.

Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients 1. The FASEB journal. 1987 Dec;1(6):441-5.

Madge T. Mini Oxford Handbook of Clinical Medicine. Nursing Standard (through 2013). 2006 Apr 12;20(31):36.

Oršolić N, Knežević AH, Šver L, Terzić S, Bašić I. Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds. Journal of ethnopharmacology. 2004 Oct 1;94(2-3):307-15.

Petrus K, Schwartz H, Sontag G. Analysis of flavonoids in honey by HPLC coupled with coulometric electrode array detection and electrospray ionization mass spectrometry. Analytical and bioanalytical chemistry. 2011 Jun 1;400(8):2555-63.

Poonam P. The biology of oral tolerance and issues related to oral vaccine design. Current pharmaceutical design. 2007 Jul 1;13(19):2001-7.

Podolsky DK. Inflammatory bowel disease. New England Journal of Medicine. 1991 Sep 26;325(13):928-37.

Robinson T, Scullion JE, editors. Oxford handbook of respiratory nursing. Oxford University Press, USA; 2009.

Read MA. Flavonoids: naturally occurring anti-inflammatory agents. The American journal of pathology. 1995 Aug;147(2):235

Rao R. Occludin phosphorylation in regulation of epithelial tight junctions. Annals of the New York Academy of Sciences. 2009 May;1165:62.

Slimestad R, Fossen T, Vågen IM. Onions: a source of unique dietary flavonoids. Journal of agricultural and food chemistry. 2007 Dec 12;55(25):10067-80.

Synnestvedt K, Furuta GT, Comerford KM, Louis N, Karhausen J, Eltzschig HK, Hansen KR, Thompson LF, Colgan SP. Ecto-5′-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. The Journal of clinical investigation. 2002 Oct 1;110(7):993-1002.

Suzuki T, Hara H. Role of flavonoids in intestinal tight junction regulation. The Journal of nutritional biochemistry. 2011 May 1;22(5):401-8.

Suzuki T, Hara H. Quercetin enhances intestinal barrier function through the assembly of zonnula occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. The Journal of nutrition. 2009 May 1;139(5):965-74.

Saeedi BJ, Kao DJ, Kitzenberg DA, Dobrinskikh E, Schwisow KD, Masterson JC, Kendrick AA, Kelly CJ, Bayless AJ, Kominsky DJ, Campbell EL. HIF-dependent regulation of claudin-1 is central to intestinal epithelial tight junction integrity. Molecular biology of the cell. 2015 Jun 15;26(12):2252-62.

Tsukamoto T, Nigam SK. Role of tyrosine phosphorylation in the reassembly of occludin and other tight junction proteins. American Journal of Physiology-Renal Physiology. 1999 May 1;276(5):F737-50.

Taylor CT, Dzus AL, Colgan SP. Autocrine regulation of epithelial permeability by hypoxia: role for polarized release of tumor necrosis factor α. Gastroenterology. 1998 Apr 1;114(4):657-68.

Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. The Journal of nutrition. 2011 May 1;141(5):769-76.

Umeda K, Matsui T, Nakayama M, Furuse K, Sasaki H, Furuse M, Tsukita S. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. Journal of Biological Chemistry. 2004 Oct 22;279(43):44785-94

Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu. Rev. Physiol.. 2006 Mar 17;68:403-29.

West JB. Pulmonary pathophysiology: the essentials. Lippincott Williams & Wilkins; 2011 Nov 30.

Wyatt JP, Illingworth RN, Graham CA, Hogg K, Robertson C, Clancy M. Oxford handbook of emergency medicine. OUP Oxford; 2012 Feb 16.

Xue X, Ramakrishnan S, Anderson E, Taylor M, Zimmermann EM, Spence JR, Huang S, Greenson JK, Shah YM. Endothelial PAS domain protein 1 activates the inflammatory response in the intestinal epithelium to promote colitis in mice. Gastroenterology. 2013 Oct 1;145(4):831-41.