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The effects of estrogen and progesterone on mechanical elements of the gastrointestinal tract and a proposed signalling pathway

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1.1 Introduction

This review focuses on two female dominant sex steroids, (1) estrogen and (2) progesterone and the influence they have on gastrointestinal health and intestinal permeability. Intestinal permeability and intestinal barrier often get used interchangeably and it is important to define these definitions correctly. Bischoff et al (2014) defines the intestinal barrier as "a functional entity separating the gut lumen from the host, and consisting of mechanical elements (mucus, epithelial layer), humoral elements (defensins, IgA), immunological elements (lymphocytes, innate immune cells), muscular and neurological elements", whilst further defining intestinal permeability as "a functional feature of the intestinal barrier at given sites, measurable by analysing flux rates across the intestinal wall". There is growing evidence to suggest that diseases such as irritable bowel disease (IBD), irritable bowel syndrome (IBS) and colitis are associated with increases in intestinal permeability, with these changes being due to a loss of intestinal homeostasis and functional impairment with barrier defects being associated with enhanced activity of pro-inflammatory cytokines (Bischoff et al., 2014). Increases in intestinal permeability have been associated with increases in systemic circulation of lipopolysaccharides, pro-inflammatory cytokines and immune cells (Xu et al., 2019) which are reported to lead to systemic immune activation, ultimately causing systemic inflammation (Zhou et al., 2017). This review will propose a possible signalling pathway involving NF-kB and pro-inflammatory cytokines on the mechanical elements in a state of disease, and the possible therapeutic benefits of the defined sex steroids.

2.1 Steroid hormones: Estrogen and Progesterone

Steroid hormones share a common basic structure of cyclopentane-perhydro-phenanthrene, a polycyclic complex of 17 carbons forming a four-ring structure. Sex steroids can be divided into three groups based on the number of carbon atoms; (1) progestins, characterised by 21-carbon atoms, (2) androgens, characterised by 19-carbon atoms and (3) estrogens, characterised by 18-carbon atoms (Taraborrelli, 2015).

Endogenous estrogen circulates the plasma in free form (active form) or bound to carrier proteins (inactive form). Estrogen exists in three biological active states, (1) 17β-estradiol (E2) (premenopausal), (2) estrone (E1) (postmenopausal) and (3) estriol (E3) (pregnancy), listed in decreasing order of potency (Parida and Sharma, 2019; Cui, Shen and Li, 2013). Estrogen is largely derived from the reduction of a 27-carbon cholesterol. In premenopausal women, synthesis largely occurs in the ovaries and placenta, with a small amount being synthesized in non-gonadal organs such as the liver, heart, skin and brain (Kwa, Plottel Blaser & Adam, 2016; Cui, Shen and Li, 2013). Estrogen synthesis also occurs in males and postmenopausal women, with production occurring from cholesterol precursors which are converted by aromatases in, adipose tissue, bone, brain and the testis for men (Parida and Sharma, 2019; Hogan, Collins, Baird and Winter, 2009; Cui, Shen and Li, 2013). The physiological range of E2 in non-pregnant women oscillates between 50 pg/ml (late luteal phase) and 1500 pg/ml (preovulatory phase) (Medina-Estrada et al., 2018) providing a range useful for researchers to analyse the effects of E2 at different physiological concentrations.

The ovaries are a major source of gonadal E2 in premenopausal women. Cui, Shen and Li (2013) describe the gonadal synthesis of E2 beginning with the synthesis of pregnenolone from cholesterol, catalysed by the cytochrome P450 side chain cleaved enzyme. Pregnenolone is then converted to progesterone (P4) by 3-β-hydroxysteroid dehydrogenase in both thecal and granulosa cells. P4 is then converted to androgens via cytochrome P450 17α-hydroxylase and 17-β-hydroxysteroid dehydrogenase in thecal cells during the follicular phase. Androgens in the granulosa cells are then converted to E2 by aromatase.

In both men and women it is documented that the adipose tissue is considered the major source of circulating extra-gonadal estrogen (Barakat et al., 2016). The major difference between extra-gonadal synthesis is the pathway involved as extra-gonadal cells and tissue are

unable to synthesis 19 carbon (C19) steroids, instead convert C19 steroids to estrogen mediated by Cyp19 aromatase. This suggests that extra-gonadal synthesis is dependent on an external source of C19 precursors and the level of local aromatase expression (Barakat et al., 2016) with tissue specific effects on aromatase expression being dependent on (1) alternative splicing mechanisms, (2) tissue specific promotors and (3) different transcription factors (Zhao, Zhoue, Shangguan and Bulun, 2016). Parida and Sharma (2019) mention that pathologies such as breast cancer are able to alter E2 synthesis by increasing production locally, with high local concentration being able to exert biological effects (Barakat et al., 2016).

3.1 Gut microbiota: Altered permeability and Estrobolome

The term gut microbiota and microbiome often get used interchangeably and it is important to define them. The gut microbiota refers to the individual microbes whilst the gut microbiome refers to the microorganisms and their metabolites. The gut microbiota consists of a variety of different species of microorganisms, including bacteria, yeast and viruses. Taxonomically, bacteria are classified according to phyla, classes, orders, families, genera and species which can be used to describe the composition of the gut microbiota along the gastrointestinal tract. According to Rhinniella et al (2019) the dominant phyla along the gastrointestinal tract are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia with Firmicutes and Bacteroidetes accounting for 90% of the gut microbiota. According to Rhinniella et al (2019) within the phyla of Firmicutes there are over 200 different genera such as *Lactobacillus, Bacillus, Clostridium, Enteroccus* and *Ruminiococcus* with *Clostridium* genera representing 95% of the Firmicutes phyla. Whilst the main genera of Bacteroidetes are *Bacteroides* and *Prevotella* (Rhinniella et al., 2019).

It is widely accepted that each individual has a unique gut microbiota profile which has been developing since birth, serving a symbiotic relationship by regulating host nutrient metabolism, maintenance of the mucosal barrier and immunomodulation, with diversity of the gut microbiota increasing with age (Rhinniella et al., 2019). The composition of the gut microbiota is known to change with age, antibiotic exposure, dietary habits, ethnicity and exercise frequency (Rhinniella et al., 2019), though this list is not comprehensive. The gut microbiome encodes over 3 million genes whilst the human genome consists of approximately 23,000 genes (Rhinniella et al., 2019). It has been reported that E2 alters the ratio of certain gut microbiota (Parida and Sharma, 2019; Zhou et al., 2019) with changes in

gut microbiota being associated with changes in intestinal permeability (Tetel et al., 2018). It is suggested that the normal gut microbiota flora prevents possible pathogenic bacterial invasion which is able to maintain intestinal epithelium integrity (Rhinniella et al., 2019). Pathogenic bacteria such as *Helicobacter pyloria* and *Salmonella* have been associated with disruptions to the tight junction (Bischoff et al., 2014). This could indicate that changes in physiological concentrations of E2 will lead to a dysbiosis in the normal gut flora leading to possible pathogenic bacterial invasion and an altered state of intestinal permeability through changes in the tight junction. Further evidence is needed to verify these claims as to what microbiota are impacted from changes in E2 and if the effects are deleterious to intestinal permeability.

It has been reported that along the gastrointestinal tract E2 is able to recycled through the concept of estrobolome, which refers to the aggregate of the total bacterial genes in the gastrointestinal tract that are capable of deconjugating E2. In the liver, E2 undergoes conjugation where it will be excreted in bile, urine and faeces. Hepatically conjugated E2 that are excreted in the bile can be deconjugated by bacterial species containing enzymes such as β-glucuronidases and β-glucosidases (Kwa et al., 2016; Parida and Sharma, 2019; Hogan, Collins, Baird and Winter, 2009). Kwa et al (2016) hypothesised that the estrobolome are able to increase systemic E2 through enzymatic activity, leading to the proliferation and development of pathologies such as estrogen-receptor positive breast cancer, which accounts for 70% of breast cancers (Parida and Sharma., 2019). The literature around the estrobolome and the effects on intestinal permeability are limited. The local effects of estrobolome on E2 may possibly lead to a dysbiosis of gut microbiota (Parida and Sharma, 2019; Zhou et al., 2019) altering intestinal permeability, though experiential evidence is needed to verify such a claim.

4.1 Gastrointestinal Estrogen Receptors

The gastrointestinal tract has been reported to contain, (1) estrogen receptor-alpha (ERα), (2) estrogen receptor-beta (ERβ) and (3) progesterone receptor (Meleine, 2014; Zhou., et al 2017). The distribution of estrogen receptors along the gastrointestinal tract suggests a possible role for sex steroids to modulate the gastrointestinal tract through receptor signalling as suggested by Barakat et al (2016) who reports that tissues expressing estrogen receptors are considered to be a target of estrogenic regulation. The majority of the effects of E2 are

elicited via canonical estrogen-mediated signalling pathways via nuclear estrogen receptors, recent evidence suggests that there are cell surface membrane receptors such as G-protein coupled estrogen receptors (Cui, Shen and Li, 2013) which are observed in pathologies such as breast cancer (Parida and Sharma., 2019). The focus of this review will be to discuss the role of E2 and P4 and how they are able to regulate mechanical elements and the possible therapeutic benefits through a proposed signalling pathway.

Along the small intestine the distribution of estrogen receptors elicits effects on cellular proliferation (Barakat., et al 2016). It is suggested that an uneven distribution of estrogen receptor isotype expression is dependent on the anatomical location along the mucosa (Barakat., et al 2016). At the base of the crypts the dominant isotype expressed are ERα whilst towards the crest the cells express the ERβ isotype (Barakat., et al 2016). It is suggested that ERα stimulates proliferation whilst ERβ opposes proliferation, with the combined net signal between these two receptors regulating cellular proliferation (Barakat., et al 2016). It was reported in a mouse model of the colon under non-pathological conditions ERβ mRNA expression was higher than ERα mRNA, though when administered with azoxymethane (AOM) and dextran sodium sulphate (DSS) to stimulate colitis there was an increase in the expression of ERα mRNA (Song et al 2018). The increase in ERα mRNA expression could be attributed to the fact that ERα is reported to increase cell proliferation of the small intestine (Barakat et al 2016) and could suggest these effects are also true for the colon, indicating a compensatory or protective mechanism to stimulate cell proliferation in the presence of endogenous E2, as primary human colon tissue is reported to predominately express ERβ (Zhoi et al., 2017). When treated with exogenous E2 mRNA expression of ERα and ERβ was reverted back to basal conditions though protein level evidence was not reported (Song et al 2018). This could suggest that estrogen-receptors are dynamically regulated to allow protective function and altering cell proliferation in response to certain pathologies such as colitis in an attempt to restore homeostasis of the colon.

5.1 Physical barrier: Mucus layer overview

The mucus layer is a complex mixture of water, salts, lipids, nucleic acids and proteins which provide a semi-permeable and selective gel, which covers non-keratinized surfaces (Witten, Samad and Ribbeck, 2018). Witten, Samad and Ribbeck (2018) state that "mucins are the main gel-forming polymer of mucus containing a large number of O-linked oligo-saccharide chains that confer a negative charge to the muccins through carboxyl and sulphate groups and also contain hydrophobic domains which aid in the self-assembly of the polymer chains". Mucins can be classed as membrane bound (MUC1, MUC3A, MUC3B, MUC4, MUC13 and MUC17) or gel forming (MUC2, MUC5AC, MUC5AB and MUC6) (Rakha et al., 2005). Different anatomical locations in the body will express different mucin proteins (Rakha et al., 2005).

The mucus layer comprises of crosslinks between the mucin proteins due to reversible hydrophobic interactions and disulphide bonds forming a polymer network, producing a mesh like structure of varying sizes ranging from 100-2000nm depending on anatomical location along the gastrointestinal tract (Witten, Samad and Ribbeck, 2018). It is suggested that the mesh size is highly heterogeneous at a given site, the heterogeneous mesh size suggests a geometric constraint imposed to particles preventing larger particles from translocating through the mucus (Witten, Samad and Ribbeck, 2018). It could be suggested that variations in the mesh structure will lead to changes in the size of the pores of the mesh, providing a logical and simple explanation on how the contents of the lumen are permitted to translocate through the mucus barrier leading to a possible increase in intestinal permeability. Witten, Samad and Ribbeck (2018) state that "little is known about the detailed molecular properties that distinguish particles that penetrate, or are rejected, by a mucus barrier" suggesting an area for future research on the permeability of the mucus layer. One could suggest changes to the mucin composition will result in changes to the size constraint imposed, leading to an altered state of intestinal permeability.

5.2 Physical barrier: Mucus layer gastrointestinal tract

The mucus layer provides innate host defence along the gastrointestinal tract and is secreted by goblet cells with the most abundant protein secreted being MUC2 (Witten, Samad and Ribbeck, 2018). Along the small intestine the mucus forms a thin monolayer that is loosely attached to the enterocytes (Hansson, 2012), with the enterocytes secreting antimicrobial peptides such as RegIIIγ which are retained in the mucus along with antibacterial peptides

produced by Paneth cells combine to reduce bacterial overgrowth in the small intestine (Hansson, 2012). Along the colon the mucus is divided into a luminal layer with larger mesh like structures, which bacteria inhabit, and a basal layer that consists of a much smaller mesh and is almost devoid of bacteria (Witten, Samad and Ribbeck, 2018; Hansson, 2012). This suggests that the size constraints imposed by the mesh layer on bacteria (Witten, Samad and Ribbeck, 2018; Hansson, 2012) as well as the continuous renewal of the mucus apically protects the colon from bacteria (Hansson, 2012), preventing disruptions in the tight junctions (Bischoff et al., 2014). In a pathological state such as colitis it is reported that the mucus layer becomes dysfunctional leading to an increase in systemic circulation of lipopolysaccharides (Schroeder, 2019), indicating an increased ability of microorganisms metabolites to come into direct contact with the surface of the monolayer of epithelial cells, possibly through alterations in the mesh like structure which may ultimately lead to systemic inflammation (Zhou et al., 2017).

5.3 Mucus layer and effects of sex steroids

Colitis is a form of IBD specific to the colonic mucosa with an unknown aetiology (Collins and Rhodes, 2006), but is thought to be due to an immunological response (Song et al., 2018). Song et al (2018) reported that in-vivo mice model mRNA expression of MUC2 protein was decreased in response to AOM/DSS treatment leading to an altered state of mucus production. Following administration of exogenous E2 at a dosage of 10 mg/kg, MUC2 mRNA expression was increased with no gender difference observed. The experimental data collected suggests that under a pathological state E2 leads to an increase in MUC2 mRNA expression (Song et al., 2018), which is reported to enhance the mucus layer suggesting a protective role of E2 possibly preventing the translocation of pro-inflammatory cytokines (Xu et al., 2019). AOM/DSS exposure was reported to lead to an increase in MUC4 mRNA expression (Song et al., 2018), though in the presence of E2, MUC4 mRNA expression was inhibited. Experimental evidence suggests MUC4 knockout mice are resistant to colitis (Das et al., 2015) suggesting a pathological link between MUC4 and colitis suggesting a reduction in MUC4 possibly improving colitis outcomes. Protein level analysis was not reported for MUC2 and MUC4 (Song et al., 2018) and the inferences made are in regard to increase in mRNA.

Diebel et al (2015) exposed HT29 cells (non-mucus producing colorectal adenocarcinoma) and HT20-MTX cells (mucus producing cells) to 90 pg/mL of E2 for up to three days to

observe the effects on MUC1 production. It was reported that HT20-MTX cells had a significant increase in MUC1 production verified by ELISA, compared to non-treated HT20-MTX cells. Run in parallel, Diebel et al (2015) also exposed HT29 and HT20-MTX cells to hydrogen peroxide for 30 minutes to induce trauma to study the protective role of E2 on oxidative stress. Oxidative damage was measured by lipid hydroperoxide release and protein carbonyl content, and cells treated with E2 were reported to have a reduction in both lipid hydroperoxide and protein carbonyl (Diebel et al 2005). These effects may be attributed to antioxidant properties of the mucus layer due to increase mucin content or other genomic and non-genomic effects of E2 as suggested by Diebel et al (2015). This suggests that in a healthy state E2 may possibly alter the mucus composition through changes in MUC1 and the protective effects observed may be due to the changes in mucus composition.

Experimental evidence both in-vivo (Song et al., 2018) and in-vitro (Diebel et al 2015) suggest that E2 stimulates mucus production through upregulation of both MUC1 and MUC2 transcription (Song et al 2018) and an inhibition of MUC4 (Diebel et al 2015) (table 1). The experimental evidence indicates that E2 serves a protective function both in a healthy state and a state of disease. It could be suggested that endogenous E2 may serve a protective function for gastrointestinal health, though further evidence would be needed to verify such a claim and look at the effects of changes in systemic endogenous E2 in response to a pathology. The dosage required to provide possible therapeutic benefits in a state of pathology is reported at a concentration dosage associated with the late luteal phase for MUC1 (Diebel et al., 2015) whilst Song et al (2018) only reported an E2 dosage of 10 mg/kg, which is indicative of a supraphysiologic dosage.

The importance of MUC2 was demonstrated in-vivo by Johannson et al (2008) who reported that in the absence of MUC2 the mucus layer was severely comprised resulting in communal bacteria being in direct contact with the monolayer of epithelial cells leading to an inflammatory response. There is growing evidence to indicate the importance of MUC2 (Song et al., 2018; Johannson et al., 2008) and experimental evidence to suggest that E2 is able to modulate mucus production (Song et al., 2018). The mucus layer may also provide antioxidant properties preventing oxidative damage and further enhancing gastrointestinal health. It could be suggested that MUC1 and MUC2 are able to restore the mesh like structure to pre-pathological conditions.

Table 1: Summary of effects of estrogen on mucin production: Experimental evidence suggest that estrogen increases MUC2 and inhibits MUC4 in a disease state and increases MUC1 in both healthy and state of oxidative stress.

Model	Treatment	Exogenous estrogen	MUC1	MUC2	MUC4	H2O2 treatment	Source
Mice	AOM/DSS (Inflammation)	Daily injections (10mg/kg) 7 days	*	Increase (ELISA)	Inhibited	*	Song et al (2018)
HT20-MTX Cell	*	Yes (90 pg/ml)	Increase (mRNA)	*	*	*	Diebel et al (2015)
HT20-MTX Cell	*	Yes (90 pg/ml)	Increase (mRNA)	*	*	Marked decrease in cell apoptosis	Diebel et al (2015)

*Not mentioned in study

6.1 Physical Barrier: Monolayer of epithelial cells

The monolayer of epithelial cells provides structural integrity of the physical barrier and regulates the translocation of luminal contents and nutrients into systemic circulation whilst preventing the translocation of pathogenic microorganisms and toxic luminal substances (Fukui, 2016; van der Giessen et al., 2019; Vancamelbeke and Vermeire, 2017). The mucosa of the small intestine is arranged in a way to increase surface area to ensure maximum absorption of nutrients with individual projections known as villi, which consist largely of absorptive enterocyte. In the luminal direction, enterocytes also contain microvilli projections, further increasing the surface area of the small intestine. At the base of the villi geographical crypts are formed which comprises of a dense collection of pluripotent stem cells that will eventually differentiate into absorptive enterocytes, goblet cells, enteroendocrine cells, paneth cells or microfold cells, all serving a unique physiological function (Vancamelbeke and Vermeire, 2017). Following cellular differentiation at the base of the crypt's enterocytes, goblet cells and enteroendocrine cells migrate apically with an expected life span of five days before being replaced with new healthy cells, supporting the integrity of the physical barrier (Vancamelbeke and Vermeire, 2017).

6.2 Paracellular pathway: Tight junctions and Adheren junctions

One of the most discussed and studied areas in the literature regarding physical barrier integrity and intestinal permeability is what connects the epithelial cells together via the paracellular pathway. The monolayer of epithelial cells are joined via junctional complexes that regulate the passage of molecules via the paracellular pathway. The junctional complex comprises of, (1) tight junctions (TJ), (2) adherens junctions (AJ) and (3) desmosomes (Vancamelbeke and Vermeire, 2017). The TJ are the apical-most adhesive complexes and consist of transmembrane proteins (claudins, occludin), peripheral membrane proteins (zonula occludens (ZO)-1, ZO-2) and regulatory proteins (Fukui, 2016; Zhou,. et all 2017; Meleine, 2014; van der Giessen et al., 2019; Vancamelbeke and Vermeire, 2017).

Tight junction regulation is an important aspect of the physical barrier and has the ability to alter intestinal permeability through altering the structure and integrity between opposite cells in the paracellular pathway. Lee, Moon and Kim (2018) suggest that "the alteration of TJ homeostasis is thought to induce the pathogenesis of several diseases and vice versa". Lee (2015) describes the structure and function of the tight junction as a complex interaction between four integral transmembrane proteins, (1) the occludins, (2) the claudins, (3) junctional adhesion molecule (JAM) and (4) tricellulin. The four integral transmembrane proteins interact with cytosolic scaffold proteins such as the ZO proteins anchoring the transmembrane proteins to the actin cytoskeleton within the cell (figure 1). The interaction between the TJ proteins to the actin cytoskeleton maintains the TJ barrier. The actin cytoskeleton is regulated by myosin light chain (MLC) activation and MLC phosphorylation by kinases such as MLC kinase (MLCK) and Rho-associated kinases. The interactions regulating the actin cytoskeleton are able to alter intestinal permeability by changing the distance between the adjacent cells in the paracellular pathway.

Another important component of the paracellular pathway which is known to maintain the physical barrier and has the ability to alter intestinal permeability are the AJ which provide adhesive contact between opposite epithelial cells. Hartsock and Nelson (2008) mention that the AJ perform a variety of functions not limited to cell-cell adhesion, such as regulating (1) the actin cytoskeleton, (2) intracellular signalling and (3) transcriptional regulation. The AJ comprise of two transmembrane spanning adhesive receptors, (1) the cadherin and (2) the nectin with the extracellular component of these proteins facilitating cell-cell interaction (Campbell et al., 2017). The cadherins comprise of over 20 members and for the purpose of this review the focus will be the epithelial cadherin (E-cadherin). Campbell et al (2017) mention that E-cadherins form trans-dimers with E-cadherins on neighbouring cells whilst being anchored to intracellular catenins, such as B-catenin, a-catenin and p-120 catenin (Campbell et al., 2017; Hartsock and Nelson, 2008). Campbell et al (2017) describe the structure of the nectins into three distinct regions, (1) the extracellular domain comprises of three immunoglobulin-like loops, (2) single pass transmembrane domain and (3) cytoplasmic tail with the extracellular component of the nectin binding with the adjacent cells extracellular component through dimerization. Whilst the cytoplasmic tail binds to the intracellular protein Afadin, anchoring the nectin to the actin cytoskeleton (Campbell et al., 2017).

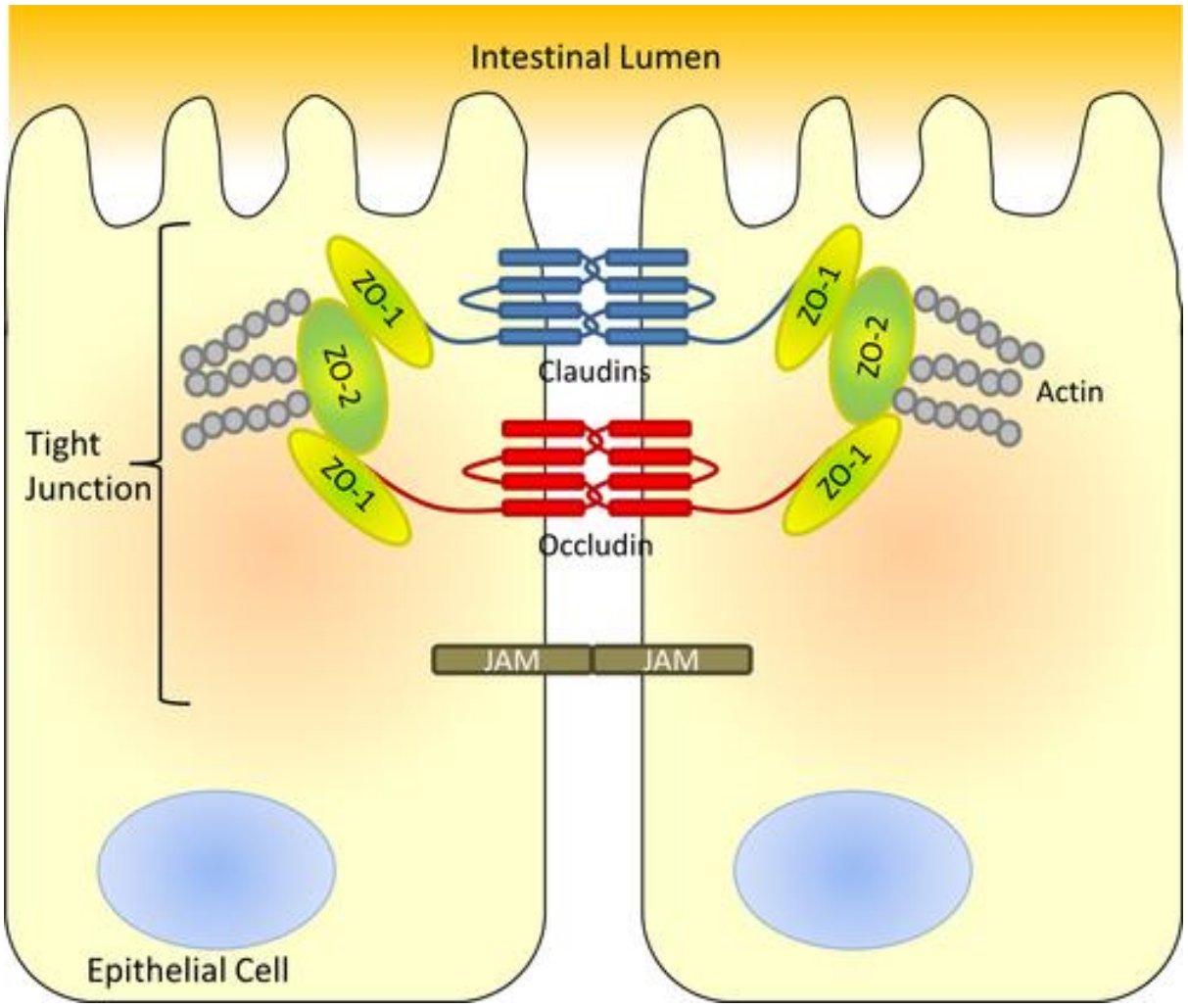

Figure 1: Schematic representation of the paracellular pathway between the enterocytes: The tight junction proteins and their respective location. Sourced from Collins et al (2017)

6.3 Paracellular pathway: Sex steroids

To study the effects the E2 or P4 researchers are able to utilise a variety of techniques such as, (1) exogenously supplying either sex steroid into a model, (2) induce postmenepause through performing an ovariectomy (OVX) and (3) verify results with the addition of an antagonists. A proxy used to assess changes in permeability can utilise transepithelial electrical resistance (TEER) over a period of time. Van der Giessen et al (2019) reported that when both Caco-2 cells and T84 cells were seeded and grown to confluency and treated with E2, P4 or a combination of both there was an increase in TEER, indicating that E2 and P4 are able to regulate of the paracellular pathway by altering the junctional complexes. In an attempt to understand the mechanism behind the change in TEER the following AJ proteins, (1) E-cadherin and (2) B-catenin were analysed via western blot with no changes reported at

protein level when treated with exogenous E2 or P4 in both a healthy state and a disease state induced artificially by tunicamycin (Van der Giessen et al., 2019). This could imply that AJ are not regulated by E2 or P4 in a disease state and that another paracellular pathway is involved, though the data around AJ and sex steroids are limited in the literature.

This paper previously mentioned that administration of E2 in a pathological state demonstrates possible therapeutic benefits through altering the mucus composition. In an attempt to model IBD in-vitro, organoid cells were generated from non-inflamed colonic biopsies and treated with tunicamycin (Van der Giessen et al., 2019). In the presence of E2, P4 or both, ZO-1, occludin and claudin-1 all showed significant increases in mRNA expression whilst claudin-2 demonstrated a significant increase in mRNA expression in the presence of E2 only (Van der Giessen et al., 2019). These findings are consistent with colitis induced by AOM/DSS treatment for male mice in-vivo (Song et al., 2018). It was reported that AOM/DSS treated female mice had an increase in ZO-1, occludin and claudin-4 mRNA expression in the absence of exogenous E2 compared to the male mice who only had an increase in ZO-1, occludin and claudin-4 in the presence of exogenous E2 (Song et al., 2017). The difference observed between genders may be due to changes in endogenous E2 in an attempt to restore TJ homeostasis, though data was not provided looking at changes in endogenous E2 (Song et al., 2018), this also suggests male mice express estrogen receptors.

In E2 deficiency induced by an OVX it was reported that mice had a significant reduction in ZO-1 mRNA (Collins et al., 2017). The experimental evidence demonstrates that E2 and P4 possibly increase a variety of TJ proteins and decrease intestinal permeability in a pathological state through altering mRNA expression of important TJ proteins such as increasing ZO-1, occludin, claudin-1, claudin-2 and claudin-4 (table 2) with E2 deficiency being associated with a decrease in ZO-1. The evidence presented implies that in a state of pathology in the absence of exogenous E2 or an E2 deficit, there are implications of the TJ proteins leading to a possible increase in intestinal permeability, possibly worsening or inducing pathologies such as IBD, IBS and colitis (Bischoff et al., 2014). In a state of pathology there is supporting evidence to indicate that E2 has possible therapeutic effects through increasing TJ proteins leading to a possible decrease in intestinal permeability.

In an attempt to study intestinal permeability in-vivo, a proxy used can be by administration of fluorescein isothiocyanate (FITC)-dextran orally. FITC-dextran when present in the

plasma is indicative of an increase in intestinal permeability as it is usually non-absorbable. After mice received an OVX, FITC-dextran was administered orally at one-, four- and eight-weeks post-surgery before mice were euthanised to analyse tissue samples (Collins et al., 2017). One week after OVX there was an associated increase in plasma FITC-dextran before being restored to pre-surgery and sham levels by week four (Collins et al., 2017). This indicates that the effect of OVX are transient and permeability was restored through other unidentified mechanisms not mentioned in the study (Collins et al., 2017). Following euthanasia tissue samples were collected from sections of the proximal duodenum, jejunum, ileum, and the distal colon and regional permeability was assayed utilising Ussing chambers (Collins et al., 2017). One-week after the OVX the ileum exhibited an increase in intestinal permeability compared to the control whilst the colon exhibited a decrease in permeability compared to the control (Collins et al., 2017). A possible explanation for difference in intestinal permeability may be due to anatomical location and cell expression, the small intestine comprises of absorptive enterocytes whilst the colon lacks absorptive enterocytes as the function of these two distinct anatomical regions are different. Previous studies (Van der Gissen et al., 2019; Song et al., 2018) may have been clearer if they introduced multiple time points or studied ex-vivo tissue samples (table 2) to observe if the possible therapeutic effects are short lived and if the effects are region-specific.

The effects of P4 have been studied to a lesser extent than E2 in the literature and highlights an area for future research. Primary human gut tissue and caco-2 cells occludin expression were analysed via a variety of different techniques such as (1) immunohistochemistry, (2) qRT-PCR and (3) western blotting at physiological concentration of P4 during pregnancy (125 ng/mL) and physiological concentrations during non-pregnancy (20 ng/mL) (Zhou et al., 2019). In-vitro occludin expression increased in a dose-dependent response to P4 which was abrogated by the presence of mifepristone, a P4 antagonist as verified by both immunohistochemistry and western blot analysis (Zhou et al., 2019). The presence of mifepristone is strong evidence that P4 is involved in the direct modulation of occludin regulation and could imply that P4 serves a possible protective role during pregnancy. Previous studies (Van der Gissen et al., 2019; Song et al., 2018) would have benefited of the inclusion of E2 antagonists to verify that the modulation was due to E2 (table 2).

Table 2: Summary of effects of estrogen and progesterone on tight junction expression: Experimental evidence suggests in a state of pathology both in-vivo and in-vitro, estrogen and progesterone lead to an increase in a variety of tight junction proteins such as ZO-1, Occludin, Claudin 1 and Claudin 2 suggesting possible therapeutic benefits by decreasing permeability. In a state of non-disease estrogen was reported to decrease ZO-1 protein level evidence.

Model	Exogenous Estrogen	Exogenous Progesterone	Ovariectomy	Antagonist	Treatment	ZO-1	Occludin	Claudin 1	Claudin 2	Implied effects intestinal permeability	Source
Organoid	10uM	No	*	*	Tunicamycin (Inflammation)	Increase mRNA	Increase mRNA	Increase mRNA	Increase mRNA	Decrease	Van der Giessen et al., 2019
Organoid	No	10uM	*	*	Tunicamycin (Inflammation)	Increase mRNA	Increase mRNA	Increase mRNA	NS	Decrease	Van der Giessen et al., 2019
Organoid	10uM	10uM	*	*	Tunicamycin (Inflammation)	Increase mRNA	Increase mRNA	Increase mRNA	NS	Decrease	Van der Giessen et al., 2019
Mice	*	*	Yes	*	*	**	**	**	**	Region specific changes	Collins et al., 2017
Mice	Daily injections (10mg/kg) 7 days	*	*	*	AOM/DSS (Inflammation)	Increase mRNA	*	*	*	Decrease	Song et al., 2018
Human colon biopsy tissue	*	20ng/mL or 125ng/mL	*	*	*	*	Increase mRNA and Immunohistochemistry	*	*	Decrease	Zhou et al., 2019
Caco-2 cell	*	20ng/mL or 125ng/mL	*	*	*	*	Increase immunofluorescence	*	*	Decrease	Zhou et al., 2019
Caco-2 cell	*	20ng/mL or 125ng/mL	*	Mifepristone (progesterone)	*	*	No change compared to control	*	*	Nill	Zhou et al., 2019
Caco-2 cell	0, 200, 2000 and 20000 pg/mL	*	*	*	*	Decrease Protein	*	*	*	Increase	Zhou et al., 2017

*Not mentioned in study

**Changes were region specific and bidirectional

Despite evidence indicating that E2 increases ZO-1 expression (Van der Gissen et al., 2019; Song et al., 2018) (table 2), other studies have found the opposite to be true. Colon tissue from postmenopausal women were collected and exposed to various concentrations of exogenous E2 (0, 200, 2000 and 20000 pg/ml) along with caco-2 cells and it was reported that ZO-1 protein level evidence decreased in response to increasing exogenous E2 (Zhou et al., 2017) at both the physiological range as previously stated and supraphysiologic range (20000 pg/mL). This suggests that as ZO-1 protein level decreases there may be an associated increase in intestinal permeability. Zhou et al (2017) mention that their study is the first to show the inhibitory effects of E2 on ZO-1 expression in primary human colon tissue. The most obvious explanations for the differences reported in ZO-1 expression is that previous studies (Van der Gissen et al., 2019; Song et al., 2018) were modelling a state of artificial pathology induced into the system (table 2) whilst Zhou et al (2017) were modelling cells which have not had any pathologies induced. Song et al (2018) reported an E2 dosage of 10 mg/kg which could be suggestive of a supraphysiologic dosage, future studies would benefit studying at a physiological range for comparability.

Assuming comparability between studies and E2 dosages, increase in ZO-1 during an induced pathology (Van der Gissen et al., 2019; Song et al., 2018) (table 2) could be attributed to pro-inflammatory cytokines. Tumour necrosis factor (α) (TNF-α) is a pro-inflammatory cytokine which forms part of the innate immune response and has been

reported to play a role in pathogenesis of IBD, leading to an increase in intestinal permeability (Al-Sadi et al., 2013; Xu et al., 2019), though other pro-inflammatory cytokines are also reported to change in response to E2 concentrations (Collins et al., 2017). Disruptions in the TJ barrier are reported to be an important pathogenic factor for development of intestinal inflammation such as Crohn's disease and colitis (Al-Sadi et al., 2013). It has been reported in-vitro that TNF-α is able to regulate MLCK gene activation (Al-Sadi et al., 2013) with MLCK being able to regulate the TJ (Lee, Moon and Kim, 2018). It is also reported in-vivo that the most obvious morphological change due to the presence of TNF-α via a MLCK-dependent pathway is the internalisation of the TJ protein occludin (Marchiando et al. ,2010). It was demonstrated in-vitro in the presence of TNF-α (25 pg/mL) caco-2 cells resulted in a marked decrease in both mRNA and protein level evidence of ZO-1 and occludin, these effects were ameliorated in the presence of adalimumab (Xu et al., 2019), which acts as an antagonist for TNF-α. The evidence presented suggests that TNF-α modulates the expression of TJ proteins (table 3), possibly leading to increases in intestinal permeability through regulation of MLCK-pathways.

Song et al (2018) reported following administration of exogenous E2 in a state of pathology induced by AOM/DSS, there was a reduction in mRNA expression of NF-kB related pro-inflammatory cytokines such as TNK-α, nitric oxide synthase and cyclo-oxygenase-2, suggesting anti-inflammatory effects of E2 through suppressing NK-kb transcription factor. Experimental evidence suggests that exogenous E2 is able to downregulate TNF-α which prevents MLCK-dependent pathways activation which has been reported to alter the TJ, possibly increasing intestinal permeability (table 3). It could be suggested that the therapeutic effects of E2 target NF-kB leading to a decrease in TNF-α (Song et al., 2018; Ito et al., 2001; An et al., 1999) further blocking MLCK gene activation preventing internalisation (Marchiando et al. ,2010) or preventing decreases protein and mRNA levels of both ZO-1 and occludin (Xu et a;., 2019) (table 3).

Table 3: Summary of effects of TNF-α on tight junction. Experimental evidence suggests that TNF-α leads to MLCK-gene activation and decrease in ZO-1 and Occludin which is prevented in the presence of a TNF-α antagonist.

Model	Exogenous Estrogen	TNF-a	Antagonist	MLCK gene activation	ZO-1	Occludin	Source
Caco-2 cells	*	Exogenous (25 pg/mL)	*	*	Decrease	Decrease	Xu et al (2019)
Caco-2 cells	*	Exogenous (25 pg/mL)	Adalimumab	*	Control level	Control level	Xu et al (2019)
Mice	Daily injections (10mg/kg) 7 days	Endogenous decrease	*		Increase	*	Song et al (2018)
Caco-2 cells	*	Exogenous	*	Yes	*	*	Al-Sadi et al (2013)
Mice	*	Exogenous (5 ug)	*	Yes	*	Decrease due to internalisation	Marchiando et al., 2010

*Not mentioned in study

Diseases such as IBD and colitis are reported to be associated with an increase in TNF-α and other pro-inflammatory cytokines (Al-Sadi et al., 2013; Xu et al., 2019). In mice exogenous E2 is able to inhibit Nf-kB leading to a reduction in pro-inflammatory cytokines such as TNF-α (Song et al., 2018), with previous in-vitro studies modelling aortic smooth muscle cells supporting these findings (Xing et al., 2012). It has been reported that E2 inhibits binding of NF-kB to the promotors of inflammatory genes (Xing et al., 2012), suggesting that E2 will lead to a reduction in pro-inflammatory cytokines. It has also been demonstrated in-vitro on caco-2 cells through an active functioning MLCK promoter region that TNF-α increases MLCK promotor activity and protein level evidence of MLCK (Ye, Ma and Ma, 2006). In-vivo mice models have demonstrated that MLCK-dependent pathways are associated with internalisation of TJ proteins such as Occludin (Marchiando et al., 2010) with in-vitro studies also reporting a decrease in ZO-1 and Occludin mRNA via a MLCK-dependent pathway (Xu et al., 2019). Figure 2 suggests a proposed signalling pathway involved in a state of disease for E2 and suggests that in a healthy state another mechanism is involved.

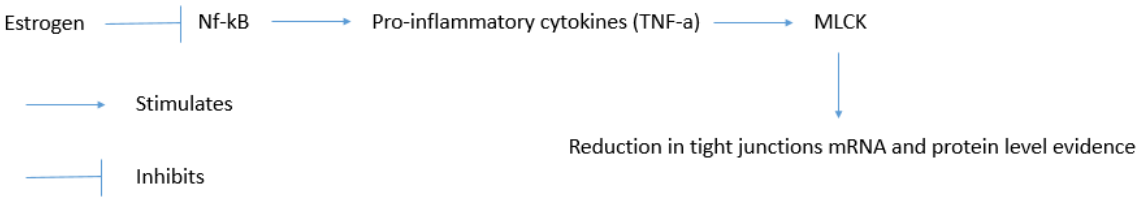

Figure 2: Proposed signalling pathway of estrogen in a state of pathology: Estrogen is able to inhibit Nf-kB promotor activation leading to a reduction in pro-inflammatory cytokines reducing MLCK activation and ultimately preventing a reduction in tight junction protein and mRNA expression. This suggests that therapeutic applications of estrogen would inhibit binding of Nf-kB inhibiting TNA-α.

8.1 Conclusion

This paper has demonstrated that in a state of pathology the physical barriers become compromised leading to an increase in intestinal permeability. Evidence of sex steroid receptors along the gastrointestinal tract suggests that E2 or P4 are able to modulate the gastrointestinal tract, with changes in gut microbiota, mucus composition, and TJ mRNA and protein level evidence being reported in response to exogenous E2 or P4, leading to both decreases and increase in intestinal permeability depending on the study. In-vivo studies demonstrate that in a state of pathology, physiological changes occur, such as the upregulation of ERα which is known to increase cellular proliferation. It could be suggested that proliferation serves as a compensatory mechanism along gastrointestinal tract, though further research would be needed to verify such a claim. It is reported that in a state of pathology the composition of mucus along the gastrointestinal tract changes due to a reduction in MUC2 and an increase in MUC4, though in the presence of exogenous E2, MUC2 expression was increased in both a state of health and diseases and MUC4 was decreased in a state of diseases. The literature around mucus composition was limited and the effects of P4 on mucus composition along the gastrointestinal tract were not explored. This suggests that E2 may provide possible therapeutic benefits through restoring the mucus composition to a pre-disease state as suggested through the increase in MUC2, and possibly decreasing intestinal permeability.

It was reported in-vivo in the presence of E2 and P4 that there was a reported increase in TEER, indicative of a decrease in intestinal permeability. The paracellular pathway comprises of the TJ, AJ and other junctional complexes not explored in this review. In the presence of E2 and P4 there were no changes reported in AJ expression, though data was limited. In response to exogenous E2 and P4 there were associated changes reported with TJ protein and mRNA expression, with inconsistencies reported. P4 was reported to lead to a dose dependent upregulation in the transmembrane TJ protein occludin in-vitro. It could be suggested that P4 provides a protective function at a physiological concentration associated with pregnancy. The effects of P4 were limited and suggests an area for future research to verify the possibility of a protective function.

The main inconsistency reported in the presence of exogenous E2 were that ZO-1 levels were reported to both increase and decrease depending on the study. In a state of pathology

exogenous E2 was reported to increase ZO-1 mRNA, whilst under non-pathological conditions exogenous E2 was reported to decrease ZO-1. Despite differences in dosages between studies a signalling pathway was proposed based on the presence of pro-inflammatory cytokines which are associated with a state of pathology. Figure 2 demonstrates a proposed signalling pathway focusing on TNF-α in pathological conditions. It was reported that E2 inhibits NF-kB both in-vivo and in-vitro in various tissue types leading to a reduction in TNF-α and other pro-inflammatory cytokines. TNF-α is reported to increase MLCK activity in caco-2 cells leading to a reduction in TJ proteins, resulting in an increase in intestinal permeability. Figure 2 suggests that in a state of pathology E2 may provide therapeutic benefit through targeting NF-kB promotor region, resulting in the downregulation of pro-inflammatory cytokines preventing the activation of MLCK activity and ultimately decreasing intestinal permeability. Possible therapeutic application of E2 should be done with caution as increases in systemic E2 have been associated with breast cancer development and altered state of gut microbiota which have been known to increase intestinal permeability, especially at a supraphysiologic dosage which would likely cause wide spread systemic effects. The signalling pathway proposed in figure 2 suggests that under non-pathological conditions another signalling pathway may be involved. Future studies would benefit from identifying alternative signalling pathways involved in non-pathological conditions and verifying the proposed signalling pathway presented in figure 2.

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