Evaluation of anti-inflammatory, immunomodulatory effects and celiac-like side effect of olmesartan medoxomil as a vitamin D receptor agonist and angiotensin II receptor blocker

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ABSTRACT: Prodrug Olmesartan Medoxomil (OM) is an angiotensin II receptor blocker (ARB) and a vitamin D Receptor (VDR) agonist. Reducing the inflammation and improving the immune system OM prevents organ damage. Angiotensin II receptor blockers (ARBs) can raise serum and tissue levels of the membrane-bound form of monocarboxypeptidase angiotensin converting enzyme 2 (ACE2). Increased ACE2 activity causes the balance in the RAAS to shift towards the positive ACE2-Ang-(1-7). Therefore It can be useful with anti-inflammatory, anti-fibrotic and anti-oxidative stress signals in the treatment of immune system diseases. OM is also known to have adverse effects, such as celiac-like enteropathy which was accepted by the FDA. The mechanism of OM's intestinal injury is thought to be the excessive consumption of the enzymes POX1 and carboxymethylenebutenolidase, which are also responsible for the the digestion of gliadin during the hydrolysis of the drug. Cell-mediated immune response and genetic predisposition are the other factors. Our histopathological findings of olmesartan-induced celiac-like enteropathy in rat intestines were increased mononuclear cell infiltration and villous atrophy. In this study these various action mechanisms of OM and its possible immun system booster effects were discussed. The findings of our rat intestines after exposure to OM-Suspension supported and correlated clinical findings of OM. In conclusion, by making extensive evaluations, OM can be a promising immunomodulator agent in immune system diseases.

KEYWORDS: ARB; vitamin-D; VDR-agonist; ACE2; Ang-(1-7); PON1; celiac-like-enteropathy; immunotherapeutic.

1. INTRODUCTION

Angiotensin II receptor blockers (ARBs) can raise serum and tissue levels of the membrane-bound form of monocarboxypeptidase angiotensin converting enzyme 2 (ACE2). Whether ACE2-enhancing drugs such as ACEIs and ARBs increase the possibility of infection and disease severity is the subject of many studies. Commonly used ACE inhibitors (ACEIs) and angiotensin-II receptor blockers (ARBs) in patients with hypertension, cardiovascular disease, and/or diabetes increase serum/tissue ACE2 levels. It has been reported that ARBs, ACEIs, RAAS inhibitors, statins, PPAR-c agonists, GLP-1 agonists and non-steroidal anti-inflammatory drugs raise ACE2 mRNA expression and/or protein levels in various tissues or plasma of animals or humans (1). Olmesartan medoxomil (OM) is an angiotensin II receptor blocker (ARB) and has pleiotropic effects unrelated to its primary mechanism of action as a prodrug (2). OM is an AT1 subtype selective antagonist of angiotensin-II receptors. Stimulating the vitamin D receptor (VDR), olmesartan, is also a VDR agonist. It reactivates the immune system, restores VDR competence, corrects irregular vitamin D metabolism and reduces inflammatory symptoms (3). Olmesartan acts similarly to 1,25(OH)2D to reduce inflammation and improve the immune system. VDR and RAS receptors are located in the same tissues. Endogenous VDR ligand 1,25(OH)2D down-regulates RAS by suppressing renin gene expression to reduce inflammation via the nuclear factor-kappa B pathway and lowers angiotensin II (a peptide involved in the inflammatory process) with the similar effect of OM (3)(4)(5). Changes in RAS efficiency are inversely proportional to changes in VDR activation and there is a feedback relationship between these systems.

Inappropriate stimulation of RAS is associated with the pathogenesis of hypertension, heart attack, and stroke(6). ACE2 is a homeostasis regulator in the cardiovascular and respiratory systems and is a component of the renin-angiotensin-aldosterone system (RAAS).

OM is also known to have adverse effects, such as celiac-like enteropathy which was accepted by the FDA (According to safety announcement UCM359496). Link between OM and celiac effect was first defined by Rubio-Tapia et al in 2012 (7)(8). This histopathological finding of celiac-like side effects are severe intestinal villous atrophy with intraepithelial lymphocytosis, increased subepithelial collagen and inflammation of lamina propria. The cessation of OM causes complete improvement of both clinical and histological features. The diagnosis of celiac disease is supported by a positive antibody test (deamidated gliadin peptide, antiendomysial antibodies and tissue transglutaminase) and symptomatic and histological response to a gluten-free diet (9)(10)(11)(12)(13)(14). The mechanism of intestinal injury of OM is thought to be a cell-mediated immune response and genetic predisposition. PON1 and carboxymethylenebutenolidase enzymes are responsible for hydrolising of olmesartan (Figure 1)(82). These enzymes are also responsible for deficient gliadin degradation in celiac patients. In patient with autoimmun disorders, OM restores VDR competence, phagocytosis causes to bacterial death; inflammation is temporarily increased by cytokine reaction to microbial endotoxins and cellular debris from dead host cells and bacteria. In this study these various mechanisms of action of OM and its possible effectiveness in immune system diseases were examined in the context of case reports and literature and then we associated with histopathological findings of our experiments in rats. During histopathological examinations, two different pharmaceutical forms of OM (Suspesion and SMEEDS forms) were evaluated, and the effect of inflammation was compared.
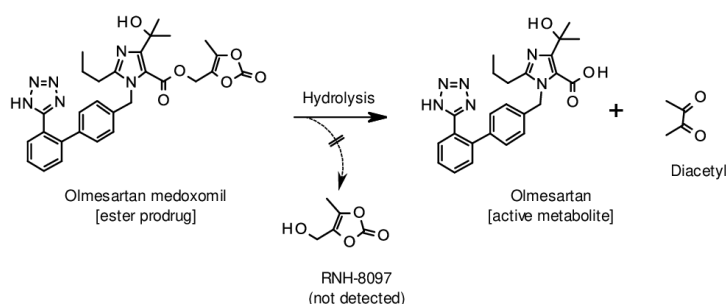

Figure 1. Hydrolisis of Olmesartan Medoxomil.

2. RESULTS

2.1. Histopathological findings

Histological studies were performed on albino 18 male normotensive Wistar Rats (160-180 g). After one month of exposure of OM-SMEDDS and OM-suspension, intestinal samples were taken from control group, OM-SMEDDS and 1.3 mg/kg OM-suspension orally administered group rats. The duodenum was used as the intestinal segment [15]. Histological examinations of rat intestine indicated that OM-SMEDDS treated rats and control group had no enteropathy findings while the OM-Suspension-treated group showed enteropathy findings with increased mononuclear cell infiltration and villous atrophy (Figure 2). OM-SMEDDS reduced the contact of OM with the intestines because of its lipophilic characteristics. This effect of SMEDDS can be explained by increased bile secretion in the gastrointestinal tract, dividing into mixed micelles, increasing lymphatic transport, and modulating enterocyte-based enzyme and carrier systems [16][17]. Throughout the experiment, SMEDDS did not cause diarrhea or weight loss compared to OM-Suspension. This finding suggests that the SMEDDS will prevent celiac-like enteropathy.

3. DISCUSSIONS

Angiotensin receptor blockers (ARBs) have been shown to modulate VDR activation through *in silico* molecular modeling [18]. OM is an angiotensin receptor blocker and also a non-vitamin D VDR ligand that reactivates the immune system, restores VDR competence, improves irregular vitamin D metabolism, and reduces inflammatory symptoms. Vitamin D is a steroid hormone that regulates the immune system. OM

regulates the vitamin D receptor and decreases the high 1,25(OH)2D to improve the function of the innate immune system [18][3].

OM has a Ki value (the binding affinity between the inhibitor and the enzyme) in the low nanomolar range similar to the Ki values of natural VDR ligands and reduces the high 1,25(OH)2D by VDR mediated effects. The up-regulated VDR transcribes CYP24A1 and CYP3A4 enzymes that reduce 1,25(OH)2D production [19] [20]. It also represses CYP27B1 (the enzyme that hydroxylates 25(OH)D to 1,25(OH)2D) so less 1,25(OH)2D is produced. A decrease in elevated 1,25(OH)2D prevents systemic inflammation. In this context OM improves glycemic control and insulin resistance by its antiinflammatory action [21]. OM also reduces serum levels of inflammatory markers; h-CRP, h-TNFa, IL-6, MCP-1 [22]. Blocking ACE OM induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity [23]. Besides, by blocking angiotensin II receptor OM increases bone mass [24][25]. OM, similar to 1,25(OH)2D, reduces inflammation and improves the immune system. VDR and RAS receptors are distributed in almost the same tissues. Endogenous VDR ligand 1,25(OH)2D downregulates RAS by suppressing renin gene expression to reduce inflammation via the nuclear factor-kappa B pathway [26]. OM similarly reduces angiotensin II (a peptide involved in the inflammatory process) [5]. Improper stimulation of RAS is associated with the pathogenesis of hypertension, heart attack, and stroke [6]. Changes in RAS activity and activation of the VDR is inversely related and have a feedback relationship. As a result, OM has dual activity in this cycle by blocking angiotensin II and stimulating the VDR and is more effective than the others. As an indicative of the Jarisch-Herxheimer reaction (JHR), olmesartan causes an increase in inflammatory symptoms in patients with autoimmune disorders and inflammatory symptoms [3][27]. OM restores VDR capability and phagocytosis leads to bacterial death. Consequently, inflammation is temporarily increased by cytokine reaction to microbial endotoxins and cellular debris from dead host cells and bacteria [28]. This immunopathology is a manifestation that OM is a VDR agonist [29][23][30]. Blocking receptors of the innate immune system can be useful in controlling infection and associated immunopathology [31]. Documented beneficial effects of OM, including its ability to reduce cardiovascular and kidney disease, prevent migraine, and reduce oxidative stress, also suggest that it may play a key role in resolving chronic systemic inflammation [32][33]. These uses of OM, which has a good safety profile, are off-label [3][34]. Microbes survive by dysregulating the VDR and slowing down immune reactivity. Chronic conditions can be improved by restoring VDR function using immunotherapy with OM as a novel VDR ligand [35][36]. As an alternative avoiding immunosuppressants and high 25(OH)D also improves immune system function [37][38][39]. Our histopathological findings of OM induced celiac-like enteropathy in rat intestines were intense mononuclear cell infiltration and villous atrophy. The mechanism of intestinal injury of OM is thought to be a cell-mediated immune response and genetic predisposition and hydrolising process with the carboxylesterases enzymes which are also are involved in the breakdown of gliadine. It is difficult to differentiate olmesartan-related enteropathy findings and histopathology from celiac disease [40]. In a systematic review performed by Burbure, findings from different case series were evaluated. In a total of 104 cases, patients who had been using OM for 1 month to 11.5 years were examined. HLDQ/ HLDQ8 gene was detected in 70%, villous atrophy was detected in 100% and IEL was detected in 70% of the cases [41]. 30% collagen sprue, 27% microscopic colitis and 41% lymphocytic or collagen gastritis were detected in cases. 95% of patients recovered after discontinuation of OM treatment. The diagnosis of celiac is supported by a positive antibody test (deamidated gliadin peptide, antiendomysial antibodies and tissue transglutaminase) and symptomatic and histological response to a gluten-free diet [42][43][44]. Genarally, villous atrophy, severe enteropathy, lupus, positive anti-TG2 deposits, increased CD3+gammadelta+ intraepithelial lymphocytes and reduced CD3− were observed in many olmesartan-induced cases and HLA-DQ2, HLA-DQ8 were positive [36]. Histopathological changes and clinical findings involving autoimmune disorders improved after discontinuation of OM. This severe enteropathy may be due to the different affinity of OM on a VDR, PPAR and CCR2b receptors that can modulate the immune system [14][11][45][46][47][48][40][7][41][49][50][51][52]. Case reports of patients taking other angiotensin receptor blockers like valsartan, irbesartan, telmisartan, eprosartan, losartan, and candesartan demonstrated a profound celiac-like enteropathy findings and villous atrophy are also exist [44][53][54][55].

Olmesartan-induced celiac-like enteropathy was determined with endoscopy and computer tomography methods by Khan et al. and Carneiro et al [56][57]. Lagana identified some histological changes related with OM in the intestine [58]. Related with pathogenesis of olmesartan-induced enteropathy, over expression of IL15 and disruption of the tight junction protein ZO-1 protein were determined in OM treated patients' doudenal biopsies as in celiac by staining anti-CD8, anti-CD4, anti-IL-15R, anti-ZO-1, anti-FoxP3, anti-psmad 2/3 [59][60][61][62]. Marthey et al. determined that OM causes severe enteropathy with or without

villous atrophy in national survey of France. Intraepithelial lymphocytes increase (IELs] was recorded in many OM celiac-like cases [63]. Severe chronic diarrhea, electrolyte imbalance, acute renal failure and significant weight loss were observed in some cases [64]. Burbure et al. have recently examined all studies about OM enteropathy and described its histopathologic differential diagnosis in his detailed study [65]. In all of these cases, clinical symptoms improved and histological changes disappeared after cessation of OM treatment [66]. According to Burbure's study, the histopathological features of celiac disease are intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy. The distinctive features of ARB-enteropathy from celiac are sometimes IEL and frequent accumulation of collagen within or near normal limits. Similarly Lagana reported that duodenal injury was associated with the use of OM [58]. The duodenal biopsy results revealed the villous atrophy and enteropathy like celiac in antitissue transglutaminase (TTG) antibody negative patient taking OM [67]. De Gateni found villous atrophy associated with OM use [68]. Although there are many case reports about this side effect, these studies represent only a certain part of the relevant cases. Many other medications except OM like mycophenolic acid, azathioprine, methotrexate, neomycin, and colchicine can cause diarrhea by increasing motility, inflammation or enteropathy and damage to the intestinal villi leading to chronic diarrhea [69][70]. A recent article based on 4 546 680 patients in the French National Health Insurance database evaluated the risk of intestinal malabsorption associated with OM, other ARBs and ACEI. When compared with other ACEI, OM is associated with intestinal malabsorption and celiac disease [71]. Case reports of patients taking other angiotensin receptor blockers (valsartan, irbesartan, telmisartan, eprosartan, losartan, and candesartan) demonstrating a profound celiac-like enteropathy findings and villous atrophy are also exist and mentioned above [72][44][73][50][49][72][74][41][53][54].
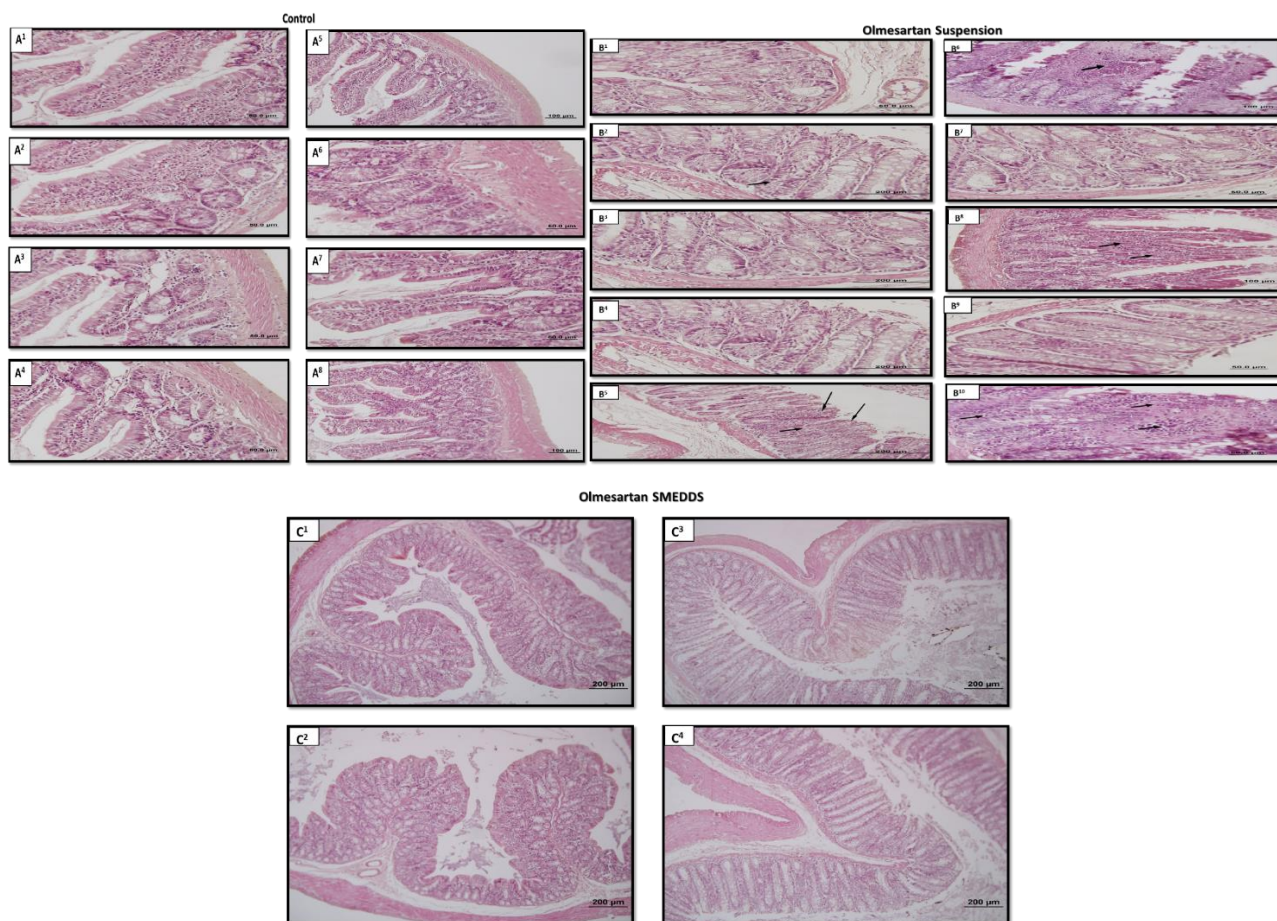

Figure 2. Images of histopathological examinations in rat duodenum after treatment with OM-SMEDDS or OM-Suspension. Formulation of OM-SMEDDS administered (C1, C2, C3, C4) and control group (A1, A2, A3, A4, A5, A6, A7, A8) intestinal imaging did not indicate enteropathy findings like celiac in contrast to the OM-Suspension administered rats. Duodenum biopsy showed Olmesartan-associated enteropathy findings, intense mononuclear cell infiltration and villous atrophy in OM-Suspension administered group of rats. Arrows indicates increased mononuclear cell infiltration (B1, B2, B3, B4, B5, B6, B7, B8, B9, B10). Scale was 50 μm, 100 μm and 200 μm.

Mechanisms during the passage of drugs through the intestinal mucosa; and the activity of the drug is related to the interaction between the drug carrier and the biological environment. In our current study, OM-SMEDDS improved the bioavailability, solubility and absorption of the drug by incorporating and encapsulating of the hydrophobic active ingredient into the oil phase, and unlike suspension, it did not harm the intestinal histology. But OM-Suspension directly interacted with intestinal mucosa and caused celiac-like enteropathy. Carboxymethylenebutenolidase (CMBL), human plasma paraoxonase (PON1) enzymes and plasma albumin are responsible for hydrolyzing prodrug olmesartan medoxomil to olmesartan and its bioactivation [75][76][77][78]. They are also responsible for the disintegration of gluten and gliadin peptides in the intestines. Therefore, excessive consumption of these enzymes involved in the hydrolysis of olmesartan caused severe diarrhea and celiac-like enteropathy [79][80][81][78][82][83]. Celiac disease is associated with oxidative damage and with significant decrease of PON1 activities [84]. The intestinal T cell response to α-gliadine in adult celiac patients is related with two immunodominant epitopes, PFPQPQLPY and PQPQLPYPQ. These epitopes are responsible for the primary inflammatory response. They are exceptionally resistant to enzymatic processing. Two brush border membrane (BBM) peptidases, dipeptidyl peptidase IV (DPP IV) and dipeptidyl carboxypeptidase I (DCP I, also known as angiotensin- converting enzyme or peptidyl dipeptidase A) are rate limiting in their digestive breakdown. Supplementation of the BBM with bacterial prolyl endopeptidase (PEP) leads to rapid cleavage of these gliadin peptides to units much smaller than the binding site of the HLA molecules. This suggests a possible enzyme supplement therapy for celiac sprue [85]. In our study, intense mononuclear cell infiltration, villous atrophy, weight loss and diarrhea were observed in the OM-Suspension administered rat group at the end of the one-month period [81][78][40][47]. Our olmesartan-induced celiac- rat- model findings supported clinical findings in this field. OM as a non-vitamin D VDR ligand also restores VDR capability and activates the immune system and reduces inflammatory symptoms. This phenomenon also explains our rats intestinal findings in terms of immune response. [28][3][86][87]. There are also many clinical studies showing that ACE2-enhancing ACEIs/ARBs used by patients with hypertension can be safely used in patients with COVID 19 [88][89][90][91][92][93][94]. ARBs increase Ang II activity on AT2R by blocking AT1R. This situation affects the conversion of Ang II to other RAS mediators such as Ang (1-7) by ACE2 acting on the Mas receptor [95]. Endogenous Ang II induces inflammation process and plays a key role in the immunomodulation of T cell responses such as activation and posterior adhesion/transmigration activity. Hence, the use of AngII/AT1 signal antagonists in therapeutic strategies to improve treatment outcomes of immune-based diseases is feasible [96]. ACE2-raising drugs increase ACE2 activity and cause anti-inflammatory, anti-fibrotic and anti-oxidative effects by ACE2-Ang-(1-7)-MasR pathway in COVID 19 patients [1]. It is reported that OM and candesartan attenuate the development of heart failure after experimental autoimmune myocarditis in rats by modulation of the ANG 1-7 mas receptor [97][98]. The importance of RAS blockade are also expressed in diabetic complications such as cardiomyopathy, nephropathy, and neuropathy [95][99]. ACE2, an ACE-related carboxypeptidase, hydrolyzes Ang I to Ang-(1-9) and Ang II to Ang-(1-7). OM increases plasma Ang-(1-7) through an increase in ACE2 expression in rats with myocardial infarction. This over-expression of ACE2 is related to a reduction in Ang II level and the cardioprotective effect of OM [100]. Quantification of immunostaining in the thoracic aorta for both ACE2 and ANG- (1-7) antibodies showed higher intensity in spontaneously hypertensive rats treated with OM as a manifestation of enhanced anti-inflammatory effect of OM in tissues [101]. ACEIs / ARBs have beneficial effects, such as lowering the risk of mortality in hypertensive patients or reducing the risk of hospitalization for COVID-19 patients with diabetes. The increased ACE2 activity causes the balance within the RAAS to shift towards the ACE2-Ang- (1-7) -MasR pathway. Anti-inflammatory effects of the ACE2-Ang-1-7-MasR pathway may explain the increased survival of COVID-19 patients using ACEI/ARBs in hypertension. [1] In another study supporting this data is Ang- (1-7) nanocarriers inhalation has been shown to induce anti-inflammatory effects and attenuated pulmonary remodeling in a mice model of allergic lung inflammation [102]. Ang (1–7) acts on Mas receptor causing vasodilatation and protects from adverse remodeling of heart and kidney. Kuba et al. reported that the RAS has a crucial role in severe acute lung injury and that the SARS-CoV receptor ACE2 has a protective role in acute lung failure. SARS-CoV Spike–mediated lung failure can be rescued by inhibition of AT1R. ACE2, a carboxypeptidase, generates Ang1-7 from angiotensin I and Ang1-9 from angiotensin II. [103][104]. VDR agonists are immunoregulators and modulate tolerogenic properties in blood myeloid but not plasmacytoid DCs [105]. In view of these data, activation of ACE2-Ang- (1-7) pathway by exogenous application of Ang- (1-7) or ACE2 or its activators would be therapeutically beneficial for immunomodulator effects. For example it has been stated anti-inflammatory agent dexamethasone is useful in the management of COVID-19 [106]. But it is also known that chronic

administration of dexamethasone reduces ACE2 levels, increases the risk of infection and plasma viral load, reduces immune function, and prevents antibody production with its immunosuppressant effect [107][108]. The high concentrations of olmesartan activates the VDR at an effective level for the activation of the innate immune response. In this case, designing drugs of ACE2-raising ACEIs/ARBs targeted for the immun diseases will be useful [102].

4. CONCLUSION

In this study various effect mechanisms of OM and its possible effectiveness in immune system diseases were discussed. Our histopathological findings of olmesartan-induced celiac-like enteropathy in rat intestines were increased mononuclear cell infiltration and villous atrophy. Hydrolysing mecanishm of olmesartan medoxomil by the enzymes like paraoxonases and carboxymethylenebutenolidase and carboxylesterase are responsible for the celiac like entetropathy. Since the same enzymes are involved in the breakdown of gliadin, long-term OM exposure causes side effects similar to celiac disease. The mechanism of intestinal injury of OM is also thought to be a cell-mediated immune response and genetic predisposition. This severe enteropathy may also be due to the different affinity of OM on a VDR, PPAR and CCR2b receptors that can modulate the immune system. Our findings of OM-Suspension exposure in rat intestines supported and correlated clinical findings of OM's celiac like enteropathy effect. But our designed OM-SMEDDS formulation did not show any enteropathy findings in the intestines. This effect was only due to the exposure of the unencapsulated pure OM in the suspension with the intestines. We proved that OM does not cause enteropathy in the intestines when encapsulated with proper lipid nano drug delivery systems specific to its solubility properties. Hence, this is a manifestation of the enteropathy adverse effect of OM is only due to local intense exposure with the hydrolising enzymes in the intestines. VDR stimulation can also be effective on severe inflammation findings in some cases. The combination of RAS blockade and VDR stimulation appears to be more effective than each one used individually. Considering VDR agonist immunogenic, ACE2-Ang-(1-7)-MasR pathway activating and ACE2-raising activities of OM it should take place as a curing agent in immun system diseases. As a non-vitamin D VDR ligand (an angiotensin receptor blocker) OM reactivates the immune system, restores VDR competence, corrects dysregulated vitamin D metabolism and reduce inflammatory symptoms. Therefore immune modulatory therapies that enhance VDR expression and activity should be considered in the clinical setting of immun diseases. In conclusion, by performing extensive evaluations, OM can be a promising immunotherapeutic and healing agent in immune diseases.

5. MATERIALS AND METHODS

5.1. Materials

Tween 80 was purchased from Merck, USA. Transcutol was granted Gattefosse, France, OM was supplied from Alembic Pharmaceuticals Gujarat, India. Span 80, Oleic acid were obtained from Sigma-Aldrich, Germany. All chemicals, reagents and solvents used in the studies were analytical grade.

5.2. Animals

Histological studies were performed on albino 18 male normotensive Wistar Rats (160-180 g). The Animals were purchased from the Central Animal Laboratory of Ege University (Izmir, Turkey) and were kept in complying with the NIH guidelines. Animals were kept polypropylene cage which is at 25±2 °C and 55±5% RH. They had freely accessed to standard diet and water, in 12 h light/dark cycle. All animal experiments were examined by the Ethics Committee of Ege University. The assay protocol was confirmed by the Ethics Committee Report of Ege University Animal Experiments, Permit Issue: 2014-073/25.02.2015

5.3. Preparation of OM-SMEDDS and OM-suspension

The experiments were carried out using our previous standardized and optimized Self microemulsifying drug delivery system (SMEDDS) with 150 nm droplet size and validated HPLC method that reported in our previous article (Komesli et al. 2019). The formulation contains 14.72% Oleic acid as oil, 16.218% Tween 80 and 16.218% Span 80 as surfactant, 32.435% Transcutol as co-surfactant and 20.41% water and OM as an active ingredient. The OM-SMEDDS was formulated at a dose of 1 mg/ml in accordance with the physicochemical properties of OM by the technique of our previous publication [109]. The OM-Suspension was obtained by suspending 0.25 g of CMC in 100 mL distilled water and adding 1 mg/ml of the active ingredient.

5.4. Administration of drug formulations

Rats were designed in three groups (n = 6). Group 1 was defined as the control group and drug was not given. Group 2 rats received a treatment at a dose of 1.3 mg / kg OM-SMEDDS and Group 3 rats received a dose of 1.3 mg / kg OM-Suspension orally once a day for a month. The dose administered was determined by considering the pharmacokinetic parameters in earlier publications and the equation 1 based on body surface area below. The OM treatment was continued for one month and the histopathological effects of formulations were analysed [15][42][43]

$$\text{Animal dose (mg/kg)} = \text{human equivalent dose(mg/kg)} \times \text{humanKm/animalKm} \qquad \text{Eq. 1}$$

Km= the surface area to weight ratios.

5.5. Histopatological examinations

After 1 month exposure to OM, the duodenal part of the intestinal samples were taken from the OM-SMEDDS, OM-Suspension-treated groups and the control group. Results were evaluated and compared histologically for celiac-like enteropathy. Histochemical examinations were made by fixing intestinal epithelial samples with 10% formol and taking sections. Samples were washed overnight under flow to remove the fixative and kept for 20 minutes in 70%, 80% and 96% ethyl alcohol and 4 different batches of acetone for dehydration, respectively. Subjected to 2 different xylenes for 30 minutes for transparency. The samples were embedded in hard paraffin after being dipped in soft paraffin 2 times, for 1 hour each. The samples were microtomized with a 5 micrometer thick rotary microtome (RM2255, Leica) [110].

5.6. Hematoxylin eosin protocol

Sections were kept overnight in a 60 ° C incubator for deparaffinization. They were exposed to three different xylenes for 20 minutes (in the oven) and twice for 10 minutes. Five different decreasing alcohol ratios were used for dehydration. The sections were rinsed with distilled water and stained with hematoxylin (01562E, Surgipath, Bretton, Peter Borough, Cambridgeshire) for 10 minutes. Sections were washed in a stream for 10 minutes to remove excess dye from tissue. It was then stained with Eosin (01602E, Surgipath, Bretton, Peter Borough, Cambridgeshire) for 2 minutes. Sections were passed through 2 series of absolute alcohol, 70%, 80%, 96%, respectively, and kept in three different xylenes to ensure 20 minutes transparency. Sections were closed with main compartments (UN 1866, Merck, Darmstadt, Germany). After staining, intestinal histopathology was visualized by electron microscopy. OM-SMEDDS, OM-Suspension, and control group images were evaluated for the presence of celiac-like enteropathy at one month.

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