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EUDRAGIT COATED CHITOSAN ORLISTAT NANOPARTICLES FOR ENHANCED ANTIOBESITY POTENTIALS – AN IN-VIVO STUDY

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ABSTRACT

Objective: Oral application of orlistat in the treatment of obesity is limited due to extensive hepatic metabolism, lack of site specific action and short elimination half-life (1-3h). Eudragit coated chitosan orlistat nanoparticles (ECONPs) were designed and evaluated to address the above mentioned limitations.

Methods: Chitosan orlistat nanoparticles were designed, optimized by Box-Behnken design. As a challenging enteric coating approach, the optimized formulation was surface coated via simple sonication and lyophilization technique using Eudragit L-100 polymer (a duodenum targeting polymer). Further ECONPs were evaluated for in-vitro characteristics and in-vivo parameters like estimation of body weight, lipid profile viz., total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) along with atherogenic index (AI) and total protein (TP) in obese rats using high fat diet (HFD) model.

Results: ECONPs produced better in-vitro characteristics with enhanced in-vitro lipase inhibition and cell viability respectively. ECONPs revealed promising in-vivo potentials with significant reduction ($p<0.05$) in elevated lipid profiles and body weights along with atherogenic index in obese rats. ECONPs also witnessed increased levels of HDL and TP.

Conclusion: ECONPs were found to be promising nanocarriers for targeted delivery and sustained release of orlistat with enhanced in-vivo antiobesity potentials.

Keywords: Obesity, orlistat, chitosan, eudragit, nanocarriers

1. INTRODUCTION

Obesity is one of the fastest-growing metabolic disorders in many parts of the world including developed and developing countries **[1]**. Obesity is closely associated with hyperlipidaemia, hypertension, atherosclerosis, non-insulin dependent diabetes mellitus and increased risk of coronary heart diseases **[2-3]**. World Health Organization (WHO) declared more than 603 million adults and 107 million children to be obese globally as of 2017 and is considered as most serious public health problems of 21st century **[4]**. A major contributing factor to the development and maintenance of obesity is excessive intake of dietary fat and triglycerides. Inhibition of pancreatic lipase and gastric lipase that leads to prevention of lipid absorption is one of the key factors for treatment of obesity **[5, 6]**. Orlistat, tetrahydrolipstatin (THL) is a specific lipase inhibitor that acts locally in gastrointestinal tract by irreversibly inhibiting pancreatic and gastric lipases involved in digestion of long chain triglycerides with subsequent inhibition of systemic absorption of dietary fat **[7-8]**. By effectively limiting dietary fat absorption, research reports proved that orlistat promotes weight loss, maintenance of lost weight, and prevention of weight regain in obese patients **[9-10]**. Due to its low water solubility and dissolution rate, only a low level of drug dissolves, hence higher doses have to be administered, but higher doses results in serious side effects like rhabadomyolysis, oily spots, borborygmi, abdominal cramps, bloating, nephropathy **[11]**.

Orlistat was explored by various techniques like micronization, pellitization, nanoemulsions, multiunit pellet systems (MUPS) and self-nanoemulsifying drug delivery system (SNEDDS) for enhanced bioavailability **[7, 12, 13]**. But none of the above approaches were aimed at delivering orlistat at its target site. The problematic nature of orlistat thus indicates the need for developing novel drug delivery carriers that delivers it at desired site for enhanced pharmacological action. Polymeric nanoparticles are of particular interest in addressing targeting problems **[14-16]**. In order to facilitate delivery of orlistat at its target site, we have designed, optimized and developed a novel orlistat formulation, eudragit L-100 coated chitosan orlistat nanoparticles in our previous study, where orlistat loaded chitosan nanoparticles was designed, optimised using 3-factor 3-level Box-Behnken experimental design (BBD) and the optimized formulation was further surface coating with eudragit L 100 (ECONPs). ECONPs produced promising results in in-vitro attributes with enhanced lipase inhibition and improved viability against selected cell lines **[17]**. However

the in-vivo efficiency of ECONPS is to be assessed, hence the present research is aimed to explore in-vivo antiobesity potentials of optimized ECONPs in obese rats using high fat diet model **[18]**.

2. MATERIALS & METHODS

Materials

Orlistat was a generous gift of M/s Aurobindo Pharma Ltd (Hyderabad, India), Chitosan, eudragit L-100, lactose was purchased from Sigma-Aldrich (Bangalore, India). Milli Q water was used throughout the study. Biochemical kits were purchased from Span Diagnostics Ltd., (India). All other chemicals and materials used in the study were generally recognized as safe with pharmaceutical grade.

Methodology

2.1 Preparation of ECONPs-Box-Behnken Design

Orlistat loaded chitosan nanoparticles (ONPs) were processed by ionic gelation method. A three factor-three level Box-Behnken design (with five centre points) was employed for the design, characterization and optimization of ONPs in our previous study. A total of 17 formulations (with varying proportions of independent variables) were prepared and evaluated for various in-vitro parameters. The effect of independent variables (amounts of chitosan (X_1), TPP (X_2) and orlistat (X_3)) on dependent variables (%Entrapment efficiency (Y_1) and %Drug release (Y_2)) was studied **[17]**. Numerical optimization technique was employed to produce optimized ONPs, further these optimized ONPs were surface coated with Eudragit L-100 polymer as per method developed by Tayel *et al*. (2015). The optimized nanoparticles (ONPs) were sonicated (5 min) in 50 ml phosphate buffer (pH 6.8) to which Eudragit L100 (0.5 % w/v) was previously dissolved. The resulting dispersion was centrifuged at 10,000 rpm for 15 min, the sediment obtained was frozen, lyophilized using lactose (1% w/v) as cryoprotectant. Thus, obtained ECONPs were characterized for particle size, electrokinetic potential, surface morphology, differential scanning calorimetry (DSC), X-ray diffraction (XRD) studies, in vitro drug release, stability studies, lipase inhibition, in- vitro cell line study (MTT assay) **[19-21]**.

2.2 In-vivo study

The in-vivo study was carried out to explore antihyperlipidemic/ antiobesity potentials of optimized ECONPs and to compare its pharmacodynamic (PD) profiles with standard preparation (O-stat). Male albino rats were procured from the animal house of Biogene laboratory Pvt. Ltd. Bangalore. Albino Wistar rats (150–200 g) were kept in polyacrylic cages maintained under standard conditions of 22±2 °C and 12 h light/dark cycle. Animals had free access to standard chow diet and

water, ad libitum **[22]**. The study was approved by the Institutional Animal Ethics Committee, (SPSP:1016/PO/Re/S/06/CPCSEA/2019/014) and animals were treated according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Pharmacodynamic studies

A total of 30 male albino rats of wistar strain weighing 150-200g were used throughout the study. The animals were randomly divided into five groups (six animals in each group, n=6). All rats were fed with commercially available normal pellet diet (NPD) and provided access to water ad libitum prior to dietary manipulation. Apart from the animals of the normal control group (group 1) which were continued to be fed with NPD, all other animals (groups 2–5) were fed with high fat diet (HFD) till end of the study. The HFD comprised of 58% fat, 25% protein and 17% carbohydrate as a percentage of total kcal **[23]**. After seven weeks feeding with HFD, body weights and lipid profiles of all the rats were assessed for their elevation and only after confirmation of induction of obesity, each group received the assigned treatment and the study lasted four weeks. The treatment groups were as follows: Group 1: NPD (Normal group); Group 2: HFD (Disease control group); Group 3: HFD & Standard product (O-stat); Group 4: HFD & ECONPs; Group 5: HFD & orlistat pure drug OPD.

2.3 Drug administration and dosing.

The standard product "O-stat" capsules were decapped and dispersed in water by sonication immediately before administration. A volume of suspensions equivalent to 10 mg/kg of orlistat was given to the animals (group-3) through oral route using an oral gavage needle. ECONPs equivalent to 10mg/kg of orlistat were re-dispersed in water by hand shaking immediately before administration and was administered orally (group-4). Similarly 2 mL of orlistat in pure form (10 mg/kg) was given orally (group-5). Once a day dosing was continued for four consecutive weeks, with simultaneous estimation of bodyweight, lipid profile, atherogenic index and total protein on 7^{th}, 14^{th}, 21^{st} and 28^{th} day **[24]**.

2.4 Blood sampling

Blood sampling was done on five occasions: once following the seven-week HFD (for confirmation of induction of obesity indicated by elevated lipid profile) right before starting treatments and remaining four times for weekly assessment of lipid profiles (on 7^{th}, 14^{th}, 21^{st} and 28^{th} day). Blood (200-300 µl) was collected from retro-orbital plexus under mild anaesthesia, into eppendorf tubes and then allowed to clot. Clotted blood samples were immediately centrifuged (Remi) at

10,000 rpm for 10 min at 4 ºC. Serum samples obtained were separated and were analysed for lipid profiling using biochemical kits and autoanalyzer **[25]**.

2.5 Assessment of biochemical parameters

2.5.1 Determination of body weights

Body weights of all groups of rats were weighed and noted prior to the commencement of treatment (with elevated body weights of group 2-5 animals fed on HFD, confirming the induction of obesity). Since then body weights of all groups of rats were measured and noted every week during the treatment period **[26]**.

2.5.2 Lipid profile

Biochemical parameters related to obesity/ hyperlipidaemia were analysed in the collected serum samples from all groups using biochemical kits (Span Diagnostics, India). Lipid parameters such as total cholesterol (TC), triglycerides (TG), low density lipoproteins (LDL), very low density lipoproteins (VLDL), high density lipoproteins (HDL), atherogenic index (AI) and total protein (TP) were estimated in the collected serum samples using standard procedures **[27]**. Atherogenic Index was calculated by using the following formula.

$$\textbf{Atherogenic Index (AI)} = \frac{\textbf{TC}}{\textbf{HDL}} \qquad (1)$$

Statistical analysis

Data was analyzed using one-way analysis of variance (ANOVA) followed Dunnett's multiple comparison test using Graph Pad Prism version 5.0. The results are presented as mean± standard error of the mean (SEM). Values of $p < 0.05$ were considered to be statistically significant.

2.6 Histopathological studies

The livers and visceral adipose tissues were excised at the end of the study and preserved in 10% buffered formalin solution for histopathological studies. Tissue was processed as per routine histological procedure such as washing, sectioning, staining with haematoxylin and eosin (H and E) and examined microscopically **[28]**.

3. RESULTS

3.1 Characterization of ECONPS

Particle size of ECONPs was found to be 534.6 nm with electrokinetic potential of +5.7 mV and possessed discrete, spherical to oval shaped loosely aggregated rough textured morphology. Solid state characterization studies of ECONPs revealed existence of characteristic peaks of orlistat within the range but with slight amorphization. ECONPs witnessed biphasic release of orlistat characterized by initial retarded (5.24 % for 2 h, P_{2h}) in pH 1.2 and subsequent sustained release (80.86 % for 24h, P_{24h}) in pH 6.8 buffer and complied with specifications of united state pharmacopoeia (USP) for enteric targeting. ECONPs were found to be stable during stability studies with enhanced lipase

inhibition and better viability against selected cell lines **[17]**.

3.2 Pharmacodynamic profile

3.2.1 Effect of ECONPs on body weight

A significant increase ($p<0.05$) in the body weight was observed in rats treated with high fat diet (HFD) when compared to normal control (group-1). Treatment with OMP and ECONPs (10 mg/kg) had shown a marked ($p<0.01$) decrease in the body weights of obese rats. ECONPs were found to be more potent than OMP in reducing body weight as shown in **Table 1**. However rats treated with OPD did not exhibited prominent reduction in the body weights as depicted in **Figure 1A**.

3.2.2 Effect of ECONPs on HDL levels

Rats of disease control group had shown a marked decrease in the HDL levels compared to normal group. Whereas, rats treated with ECONPs witnessed significant increase ($p<0.01$) in HDL levels compared to rats treated with OMP and OPD **(Table 1 and Figure 1B)**.

3.2.3 Effect of ECONPs on serum TG and TC

Rats treated with HFD shown a statistically significant ($p<0.05$) increase in the serum TG and TC levels. Treatment with OMP, ECONPs (10 mg/kg) have shown marked ($p<0.01$) decrease in the serum TG and TC levels where ECONPs were found to be equipotent to OMP as shown in **Table 1 and Figure 1C**.

3.2.4 Impact of ECONPs on serum LDL levels

ECONPs significantly ($p<0.01$) reduced LDL levels of obese rats compared to OMP. LDL levels were significantly ($p<0.05$) increased in rats fed with HFD compared to rats maintained on normal pellet diet (NPD) as shown in **Table 2**. However prominent decrease in LDL levels was not observed in rats treated with OPD **(Figure 2A)**.

3.2.5 Impact of ECONPs on serum VLDL levels

VLDL levels were significantly ($p<0.05$) raised in rats of disease control (group-2) compared to normal control group rats. Treated rats with OMP, ECONPs and OPD had shown a marked ($p<0.01$) decrease in the serum VLDL levels. However compared to ONPs and OPD, ECONPs were found to be superior in reducing VLDL levels in rats as shown in **Table 2 and 2B**.

3.2.6 Role of ECONPs on serum Total proteins

In addition to the lipid parameters, total protein levels were also estimated in the serum samples of rats of all groups as a measure of liver function. Total proteins levels were significantly ($p<0.05$) reduced in HFD rats compared to rats of normal group. On the other hand, Total protein levels were increased and maintained (as that of normal group rats) in the rats treated

with ECONPs and OMP, where ECONPs were equipotent as that of OMP **(Table 2)**. However, OPD was unable to raise/restore total proteins levels and resembled disease control rats as shown in **Figure 2C**.

3.2.7 Impact of ECONPs on Atherogenic index (AI)

Atherogenic index, a consequence of obesity/ hyperlipidaemia/, was drastically and significantly ($p<0.05$) elevated in rats fed with HFD. Whereas ECONPs and OMP were found to be effective in reducing AI compared to OPD as shown in **Table 2**. However ECONPs were equipotent as that of OMP in reducing AI **(Figure 2D)**.

3.3 Histopathological studies

Histology of liver tissues of all groups of rats is depicted in **Figure 3** where hepatocytes of rats treated with ECONPs resembled with that of hepatocytes of untreated groups (normal rats). Similarly histomicrographs of adipose tissues of all rats are elucidated in **Figure 4**, where the adipocytes of ECONPs treated rats retained normal architecture.

Table 1: Effect of ECONPs on Body weight, HDL, TG and TC at the end of 28th day

S. No	Group	Body Weight	HDL	TG	TC
1	Normal control	237.6±2.05	49.0±3.26	114.0.±2.16	98.33±1.24
2	Disease control	345.0±2.44*	19.0±3.26*	177.0±2.16*	179.66±1.69*
3	OMP (10mk/kg)	247.6±2.05**	49.66±0.9**	114.0±1.3**	112.0±2.44**
4	ECONPs (10mk/kg)	242.0±2.16**	60.3±1.24**	108.6±2.86**	108.3±2.49**
5	OPD (10mk/kg)	256.0±2.44**	42±3.26**	134.0±3.55**	127.0±2.16**

*p<0.05 (when compared with control), **p<0.01 (when compared with disease control)

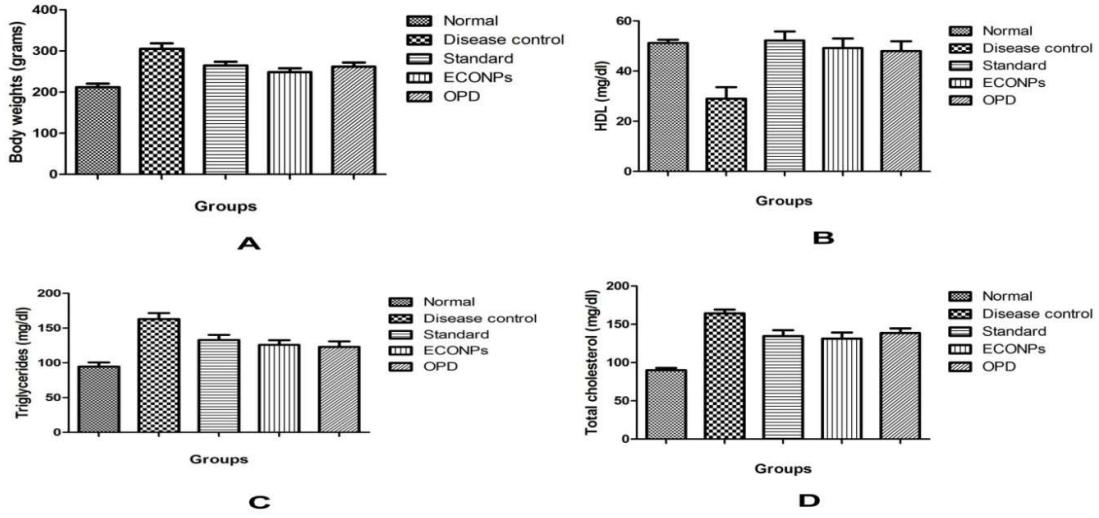

Figure 1: Body weights (A), HDL (B), TG (C) and TC (D) profiles of Normal control, Disease control; OMP, ECONPs and OPD treated groups

Table 2: Effect of ECONPs on LDL, VLDL, TP and AI at the end of 28th day

S. No	Group	LDL	VLDL	TP	AI
1	Normal control	72.0±1.63	31.0±1.63	7.60±0.43	1.83±0.62
2	Disease control	142.66±1.24*	71.33±2.05*	2.83±0.65*	9.46±0.24*
3	OMP (10mk/kg)	62.66±1.69**	41.33±2.86**	8.1±0.32**	1.7±0.16**
4	ECONPs (10mk/kg)	58.33±2.05**	36.66±1.69**	8.53±0.32**	1.8±0.32**
5	OPD (10mk/kg)	81.66±7.71**	49±1.63**	4.16±0.24**	2.13±0.53**

*p<0.05 (when compared with control), **p<0.01 (when compared with disease control)

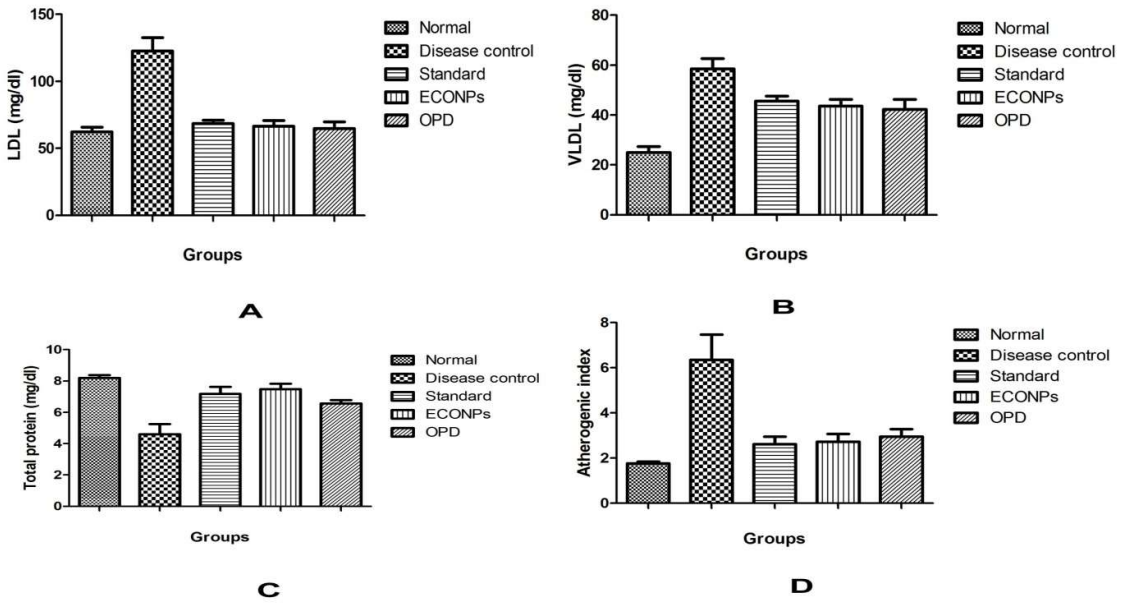

Figure 2: LDL (A), VLDL (B), TP (C) and AI (D) profiles of Normal control, Disease control, OMP, ECONPs and OPD treated groups

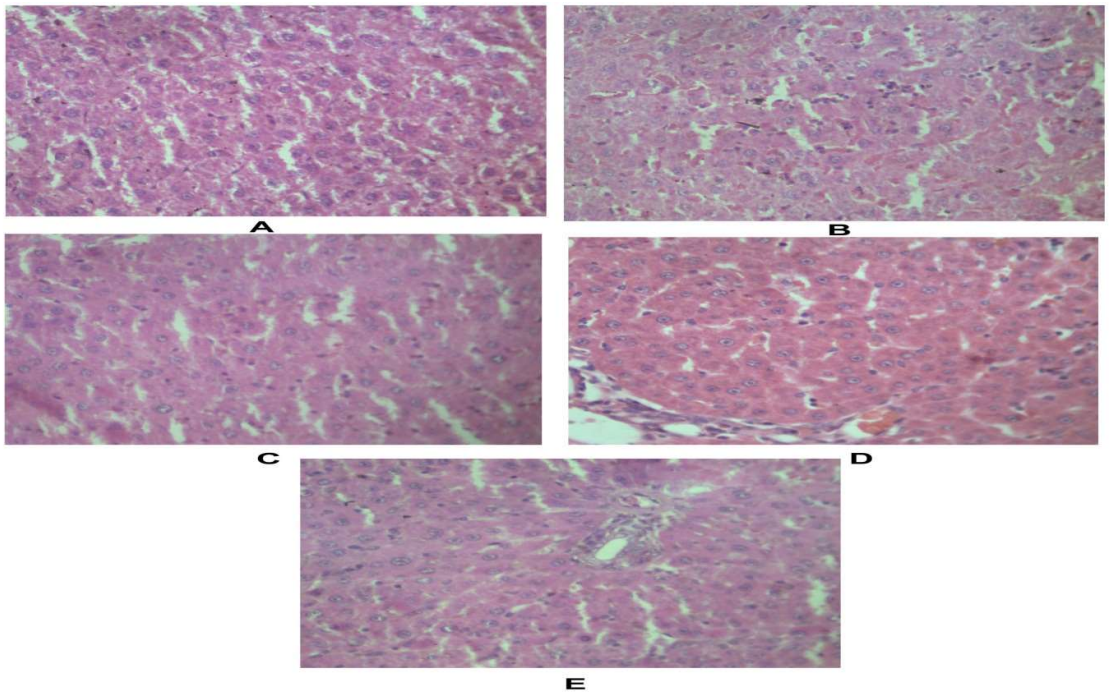

Figure 3: Histomicrographs showing histopathological changes in Livers of Normal control (A), Disease control-HFD (B), OMP (C), ECONPs (D) and OPD (E) treated rats

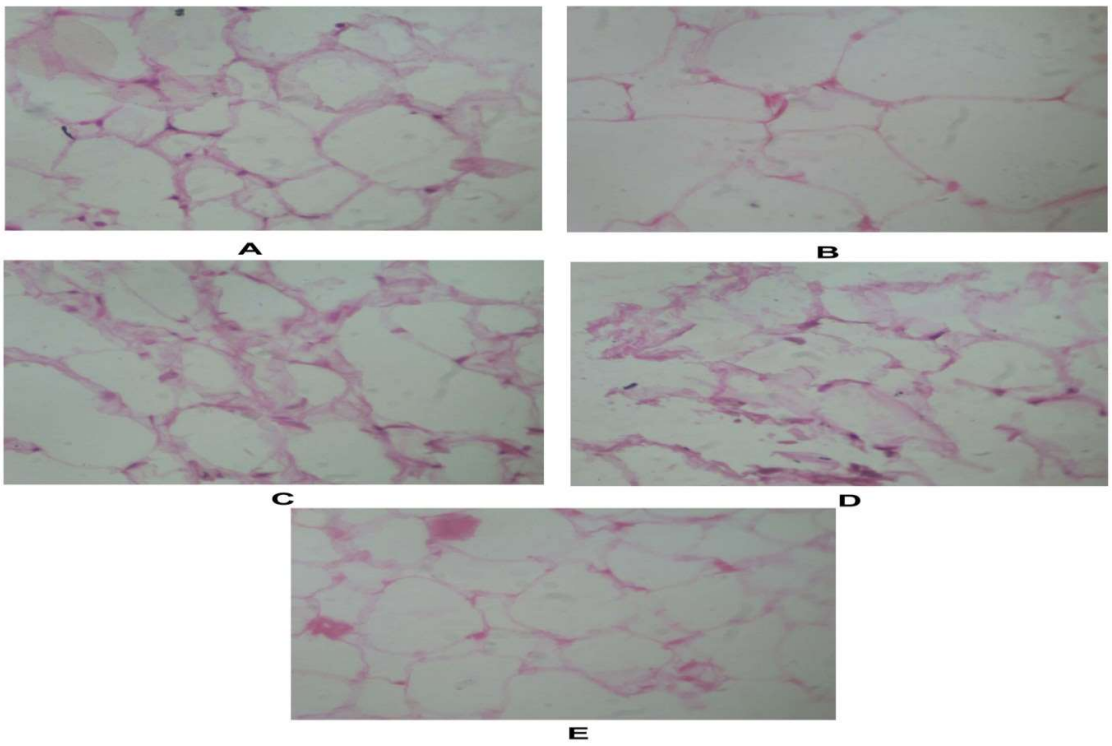

Figure 4: Histopathological investigations of adipose tissues of Normal control (A), Disease control-HFD (B), OMP (C), ECONPs (D) and OPD (E) treated rats

4. DISCUSSION

Design, optimization and surface coating of ONPs with eudragit L-100 (ECONPs) was a successful approach for targeted drug delivery of orlistat. ECONPs also proved their efficiency in in-vivo studies as signified by the results obtained. The most critical challenge for targeted drug delivery is to preserve the designed formulation during its transport through the stomach and upper part of the small intestine. Such strategies would assure not just direct treatment at the target site, but also but also the possibility of a reduction in administered dose and any associated systemic adverse effects. The key factor and rate determining step as indicted in our study is to prevent the absorption of dietary fat and this was successfully achieved by ECONPs where the release of orlistat (biphasic pattern) was anchored at desired site (duodenum) there by action of duodenal/intestinal lipases was effectively inhibited. The gastro-retentive potentials of ECONPs met the USP (2013) requirements for enteric-coating (P_{2h} of less than 10%). Furthermore, the sustained drug release profiles for 24 hours at pH 6.8 were attributed to protonation or deprotonation of Eudragit L100 at different pH settings. The carboxylic groups of Eudragit L100 were protonated at pH levels lower than the pKa of methacrylic acid, which is 4.23. The enteric surface-coated nanoparticles were

protonated in a simulated acidic stomach environment (pH 1.2) to reduce surface charges and electrostatic repulsions. Thus, established Vander Waals forces aggregated the ECONPs, and conversely carboxylic groups were deprotonated at simulated intestinal milieu (pH 6.8), resulting in biphasic orlistat release **[29 - 31]**.

ECONPs showed their promise in significantly reducing and controlling the elevated body weights of obese rats throughout the study. ECONPs also showed their promise in significantly reducing the elevated lipid profile and this could be attributed due to successful inhibition of absorption of dietary fat. Rats fed with HFD (group-2) witnessed marked elevation in their lipid profile compared to rats maintained on normal pellet diet (group-1). On the other side, ECONPs were found to be more potent compared to standard product (O-stat) in reducing the elevated lipid levels of obese rats. ECONPs reduced atherogenic index of obese rats and finds their application in reducing cardiovascular risk factors associated with obesity. However pure form of orlistat (group-5) failed to reduce the elevated lipid profile and body weights in obese rats and this could be due to inadequate concentrations at the target site. Histopathological features of liver and adipose tissues are depicted in **Figure 3 and Figure 4**. In rats of normal control group, the liver was seen with normal architecture without any inflammation, fibrosis, fatty changes and necrosis. On contrary, the liver tissue of rats fed with HFD (group-2) evidenced altered architecture where the hepatocytes with fatty vacuoles, mononuclear cell infiltration, facilitated granular degeneration was observed. However, no changes/abnormalities were observed in liver histology of rats treated with ECONPs, also regeneration of cells (indicated by binucleated cells) was evidenced with ECONPs treated rats. Rats treated with OMP exhibited normal architecture without any alterations. However, regeneration of cells was not up to the mark with OPD treated rats as depicted in **Figure 3**. The histopathological examination of adipose tissue revealed that the adipocytes were spherical to polyhedral shaped cells with eccentric nucleus in rats fed with NPD. Whereas, swollen adipocytes with thick membrane and slight eosinophillic granules with cytosteato necrosis was observed in rats fed with HFD. Adipocytes with spherical and eccentric nucleus were seen in rats treated with ECONPs and OMP and resembled with the normal adipocytes. On the other side, adipocytes were slightly swollen with OPD treated rats, the histopathological observations of adipose tissues are depicted in **Figure 4**.

5. CONCLUSION

Eudragit coated chitosan orlistat nanoparticles (ECONPs) had revealed promising in-vivo antiobesity potentials. A significant reduction in the serum TG, TC, LDL, VLDL, AI, body weights and a significant increase in the levels of HDL and total protein was achieved, thus indicating the potential use of ECONPs in the treatment of obesity and in other associated disorders like atherosclerosis. ONPs designed, optimized by BBD using ionic gelation method and subsequent surface modification with eudragit L-100 (ECONPs) was a promising approach for targeted drug delivery with the results achieved. ECONPs found to be superior and equipotent as that of OMP at lower doses and find their application in the effective management of obesity. Further pharmacokinetic and pre-clinical studies using suitable models are essential to prove the antiobesity potentials of ECONPs.

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Disclosure statement

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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