Histochemical localization of acidic and neutral lipids with phospholipids in small intestine of mice after beta agonist fenoterol administration

POOJA SHARMA*

SUSHMA SHARMA**

Abstract

Histochemical study of the small intestine of Balb-C strain mice was carried out at microscopic level. Anatomically, the small intestine has a serial relationship with the liver relative to the absorption and is the anterior organ. The amount of an orally administered drug that reaches the systemic circulation can be reduced by both intestinal and hepatic metabolism. The metabolism of drugs before entering the systemic circulation is referred to as first-pass metabolism. The three regions of the small intestine share a common histological pattern. Their wall, from inside outward, is composed of the mucosa, the submucosa, the muscle layers, and the serosa **(Thomas, 1988)**. The serosa is an extension of the peritoneum and consists of a single layer of flattened mesothelial cells overlying some loose connective tissues. The muscularis has an outer longitudinal layer and an inner circular layer of muscles. The submucosa is composed of a network of loose connective tissue rich in small blood vessels, lymphatics, and nerve plexus. The mucosa has three components: a superficial lining of epithelium, the lamina propria, and the muscularis mucosa. The epithelium, the innermost layer of mucosa facing the lumen of the bowel, consists of a single layer of columnar epithelial cells (enterocytes) which line both the crypts and the villi. The villus height decreases from the jejunum to the ileum. The total mucosa thickness varies from 530 to 900 mm **(Thomas, 1988).** The phospholipids are arranged in a bilayer, with their polar, hydrophilic phosphate heads facing outwards, and their non-polar, hydrophobic fatty acid tails facing each other in the middle of the bilayer. Furthermore, the

activity of membrane proteins is directly affected by their interaction with membrane phospholipids **(Dowhen *et al*., 2009).** A change in the phospholipid profile of membranes has long been implicated in the development of diseases **(Barenholz and Thompson, 1980)**. Mucosal phospholipids rapidly take up free fatty acids from the lumen of the small intestine. Histochemical analysis showed that goblet cells have acidic and neutral lipids. Alterations in the phospholipid level and acidic and neutral lipids in small intestine were studied from 7 to 28 days in normal and drug treated mice.IN recent years several workers have studied by histochemical as well as quantitative methods the various metabolites and enzymes of the fat body of the mice. However, no information is available on in this tissue. Here, we report the results of a histochemical study undertaken to demonstrate acidic and neutral lipid with phospholipid activity in the fat body of the mice. We interpret the decrease in acidic and neutral lipid with phospholipid activity as an adaptation of the enteric mucosa to maintain the absorptive function of small intestine.

Key Words: Beta –agonist, Fenoterol, Villi, Epithelial cells, lumen.

*DEPARTMENT OF BIOSCIENCES, SUMMERHILL, SHIMLA, INDIA

**DEPARTMENT OF BIOSCIENCES, SUMMERHILL, SHIMLA, INDIA.

INTRODUCTION

Smooth muscles display remarkable variations in their functions which range from characteristics peristalitic movements of alimentary tract, mesentaries and rhythmic contractions of uterine wall **(Dilley *et al*., 1987; Owens, 1989; Shwarts *et al*., 1990; Thyberg *et al*., 1990).** The extreme variations in the functions of smooth muscle tissues emerge as a consequence of an inherent structural and functional plasticity which enable the tissues to undergo adaptive responses comprise essentially of variation in smooth muscle tissue mass. It may include myointimal hyperplasia, myointimal hypertrophy, change in the wall thickness, alteration in the wall lumen ratio and also changes in the dedifferentiated state of constituent muscle cells **(Dzau and Gibbons, 1993; Lowell, 1993; Shwartz and Liaw, 1993).** Anatomically, the small intestine has a serial relationship with the liver relative to the absorption and is the anterior organ. The amount of an orally administered drug that reaches the systemic circulation can be reduced by both intestinal and hepatic metabolism. The metabolism of drugs before entering the systemic circulation is referred to as first-pass metabolism. Three of the small intestine regions are not anatomically distinct, although there are differences in their absorptive and secretary capabilities. The three regions of the small intestine share a common histological pattern. Their wall, from inside outward, is composed of the mucosa, the submucosa, the muscle layers, and the serosa **(Thomas, 1988)**. Fenoterol is used in the form of fenoterol hydrobromide as intravenous, oral or inhaled aerosol formulations. By the oral route, fenoterol 5mg (4-8 times daily), has been shown to be effective **(Dudenhausen *et al*., 1989)**. Fenoterol is a selective β_2- adrenoceptor agonist that has been in clinical use for decades. Fenoterol is more selective for β_2 adrenoceptors and its action is rapid **(Nelson, 1988; Jenne and Tashkin, 1993)**. Peak plasma level generally occur within 20 to 90 minutes, however 75% of maximal effect is achieved within 5 minutes **(Nelson, 1988).** Fenoterol being stimulator of both β_1 and β_2 adrenoceptors is found to be more myotoxic. Histochemical analysis showed that the goblet cells have acidic and neutral mucosubstances. Present study demonstrated for the first time that oral administration of fenoterol can enhance muscle apoptosis and hypertrophy. Although the improvements in regenerating fiber size and muscle function associated with a single dose of fenoterol were emphasized.

MATERIALS AND METHODS

Adult Swiss albino male mice of Balb- C strain weighing 25-30g were procured from Central Research Institute (CRI), Kasauli, Himachal Pradesh. They were housed in polypropylene cages under controlled conditions of temperature and light (24 ± 2^0C; 16 hr day light) and fed upon Hindustan lever pellet diet and water *ad libitum*. All experimental procedures were conducted after the approval of Institutional Animal ethics committee, Himachal Pradesh University (IAEC /BIO/4-2006), Shimla.

Mice were randomly assigned into two independent groups: One group containing normal mice served as control and the other group included mice as treated groups. Animals of second group were given daily oral administration of fenoterol (1.5 mg/ kg body wt) for 28 days).

Tissue Harvesting

Animals were sacrificed at 7, 14, 21 and 28 days by cervical dislocation. Small intestine (duodenum, jejunum and ileum) were immediately excised. At least 4-6 animals from each group were sacrificed at each stage.

Cytochemical localization of the lipids was achieved by histochemical techniques. Qualitative changes in the distribution of phospholipids, acidic lipids, neutral lipids, fatty acids and lipase activity in the tissue sections from duodenum, jejunum and ileum were evaluated by means of histochemical studies. Details of different histochemical methods were as follows.

(a) Acidic and Neutral Lipids. Histochemical localization of acidic and neutral lipids was demonstrated by the Nile Blue Sulphate staining method of **Cain (1947)**. Fresh frozen, hand cut sections (15 - 20 µ thick) were mounted on the clean glass slides. These were then thawed and air dried. The tissue sections were then fixed in calcium formol for one hour and then transferred to distilled water. Sections were stained in 1% nile blue sulphate at 60^0C. Differentiation of the sections was achieved in 1% acetic acid at 60^0C for 30 seconds. Double stained sections were washed in distilled water and were mounted in glycerol jelly.

(b) Phospholipids. The distribution of phospholipids in tissue sections was demonstrated by the acid – haematin method of **Baker (1946)**. Fresh frozen, hand cut transverse muscle sections of 15 - 20 µm thickness were mounted on clean glass slides and allowed to thaw and made air dried. Calcium formol was used to fix phospholipids of tissue sections. After the fixation, sections were washed in distilled water and transferred to dichromate calcium solution for 1 hour at 60^0C for the chromation. Chromated sections were rinsed in distilled water and then stained in acid haematin for 45 minutes at 37 ^{0}C. Stained sections were washed thoroughly in distilled water. Differentiation was achieved in Borax ferricynide solution for 18 hours at 37^0 C. The tissue sections were finally washed in distilled water and mounted in glycerol jelly.

RESULTS

a) Acidic and Neutral lipids

Nile blue staining method of **Cain (1947)** was employed to examine the distribution pattern of acidic and neutral lipids in tissue sections. Nile blue is a metachromatic dye which stains acidic lipids or fatty acids blue and neutral lipids pink. A hydrolysed solution of nile blue sulphate comprises of partly a blue salt and partly a red free base. The distribution pattern and sites of staining products hence, allow a convenient distinction between the two types of lipids in tissue sections. Histochemical localization of neutral lipids by nile blue stain is confirmed by pink colored dye distribution.

Duodenum

The lamina propria, the intestinal glands and enterocytes showed moderate, diffused as well as granular acidic and neutral lipid localization in normal duodenum. The muscularis mucosa was stained deep pink. Maximum localization was observed in the enterocytes, but lamina propria showed reduced acidic lipids as compared to enterocytes. The amount of acidic lipids was high in the villi and intestinal glands (Fig. 1).

A decrease in the lipid localization was noticed after 7 days of fenoterol treatment. The mucosa showed decrease in neutral lipid content as compared to normal duodenum mucosa. It

was lightly stained with pink colour. The intestinal glands showed high acidic lipid accumulation (Fig.2).

The microscopic examination of the duodenum exhibits luminal surface which is completely covered by a number of leaf like projections called villi. Hypertrophy of the villi and intestinal glands was observed after 14 days of drug treatment. The villi and intestinal glands contained blue coloured stained acidic lipids, while the mucosa contained neutral lipids stained with pink colour (Fig.3)

Bursting of the tip of the villi takes place after 21 days of fenoterol treatment. There was a decrease in the acidic lipid content in the villi (Fig.4). After 28 days stage of drug administration, the villi get elongated as compared to duodenum. Goblet cells were noticed in the mucosa, they appeared pale or empty due to loss of their contents. The neutral lipids are less abundant in the mucosa and were faintly stained with pink colour. Interfibriller spaces were almost completely free of any acidic lipids. Decline in the interfibriller staining was thus characteristically observed. Administration of fenoterol possibly stimulated the release or induced a mobilization of acidic lipids from intracellular to intercellular spaces. Thus, at 28 days stage the acidic and neutral lipids were considerably reduced in the villi, intestinal glands and in the mucosa (Fig 5).

Jejunum

The acidic and neutral lipids in the normal jejunum were moderate, diffused and granular in the lamina propria, enterocytes as well as in the intestinal glands. The muscularis externa was deeply stained with pink colour while the acidic lipids were stained blue (Fig. 6).Elongation of the villi was observed after 7 days of fenoterol treatment. The villi stained with blue colour showed presence of acidic lipids in small intestine. Intercellular spaces were also appeared which were devoid of acidic lipids (Fig. 7). The stained sections showed abrupt elongation of the villi after 14 days of drug administration. Lamina propria was lightly stained with blue colour while the muscularis externa was stained pink thereby depicting the presence of neutral lipids. The submucosa was without stain while the crypts and villi were greatly hypertrophied. The mucosa, submucosa and muscularis externa were shown clearly (Fig. 8). At 14 days stage

longitudinal and circular muscles were noticed in muscularis mucosae (Fig.9). There was complete absence of Brunner's gland in submucosa. Disruption of the villi was seen and bursting of villi was noticed after drug treatment at 21 days stage. Crypts villus architecture was completely disrupted. The villi were faintly stained with blue colour (Fig.10).

Plicae circularis were clearly visible in the treated jejunum after 28 days stage (Fig.11). Plicae consist of core of submucosa and the overlying mucosa. They have a semilunar, circular or spiral form extended about one half to two thirds around the circumference of the lumen. Plicae in the jejunum tend to be taller and thinner and were more frequent.

Ileum

Ileum is the distal part of small intestine. Acidic and neutral lipids in this part were diffused and granular. The muscularis externa was stained faintly with pink colour while the villi were stained with blue colour. They were compactly arranged (Fig.12). Villus coalescence was observed after 7 days of fenoterol treatment. Muscularis externa stained with pink colour showed the presence of neutral lipids. Villi were blue coloured showing the presence of acidic lipids without any spaces. The microscopic architecture of ileum at 14 days stage of drug administration showed that several villi with an abnormally large and hypertrophied structures in ileum region of small intestine. The basal lamina along the base of enterocytes was damaged. These changes established that fenoterol results in hyperplasia. After 21 days of fenoterol treatment, the villi get ruptured. More goblet cells appeared which were without stain. Decrease in the concentration of acidic lipids in the villi as they are faintly stained with blue stain. Muscularis externa stained pink (Fig.13). Crypt abscess and branching of crypts were observed. Fusion of the villi was observed after 28 days of drug administration (Fig.14). The intercellular space in between the villi was devoid of acidic lipids. The villi contained very less acidic lipids at 28 days stage. Striated border of epithelium were clearly seen without any stain. Basement membrane get shrunken away from the epithelium.

Phospholipids

Acid haematin (AH) staining employed as a tool to assess qualitative changes in the phospholipid content of normal intestine. Baker's acid haematin technique results in the staining

of phospholipids as intense brown or black deposits in the tissue sections. The unsaturated lipids appear brown. Examination of muscle sections revealed a distribution of pale brown or brownish yellow coloration. The lipids were discernible as dark brown granules scattered over a more soluble pale yellow coloured background. Histochemical studies were intended to understand the lipid distribution pattern in the normal and fenoterol treated small intestine of mice. The histochemical reactions were assessed visually by the intensity of the particular staining reaction.

Duodenum

The tissues from the duodenum were sectioned for histochemical characterization of phospholipids in the lamina propria, tunica submucosa, tunica muscularis and serosa layers. The intestinal glands were stained with deep brown colour (Fig.15). The lamina propria was stained with light black color. Lacteals were also appeared without stain.

The villus get hypertrophied, phospholipids get decreased in the villi after 7 days of drug treatment. The intestinal glands were stained yellow which showed decrease in phospholipid content. The muscularis externa, mucosa and serosa layers showed decrease in the phospholipid content as these were faintly stained with brown colour after 7 days of fenoterol administration. The lamina propria was stained light black in colour (Fig.16). Majority of the villi were intact and upright but distorted and tilted. Villi with lost tips were also frequently observed which showed significant increase in height. Hypertrophy of the villus was observed after 14 days of drug treatment. The space between villus gets decreased. The lamina propria was stained with light black colour. Duodenal mucosa demonstrated hypertrophy. Villus hypertrophy was observed after 21 days of fenoterol administeration. The phospholipid content get decreased and showed very light staining. The lamina propria was faintly stained with black color. There was no space in between the villi . Lact Histochemical localization revealed presence of the muscularis adventitia followed by muscularis externa after 28 days of drug treatment. The longitudinal and circular muscles were observed distinctly. The colescence of the villus was observed (Fig.17).The lamina propria was deeply stained with black color. The epithelium of the villus was not observed clearly.

Jejunum

The jejunum of normal mice illustrated the presence of muscularis externa, sub- mucosa and mucosa. It was stained light brown in colour thus showing less amount of phospholipid content. Lamina propria was stained light black . Mucosal inflammation was observed after 7 days of fenoterol treatment. Hypertrophy in the villus structure was also observed. The architecture of villus was disrupted completely after 14 days of drug treatment. Splitting of the tip of the villus was clearly observed. The lacteal was devoid of stain. Epithelium damage after drug administration was clearly visualized (Fig. 18).

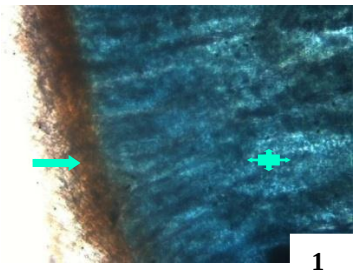
The complete loss of phospholipid content was observed in the muscular adventitia after 21 days of drug administration . Loss of phospholipids was also appeared in the intestinal glands. Hypertrophy of the villi was observed clearly. Lamina propria was again devoid of phospholipid content. Epithelial damage was also seen in the tissue. The villi get extremely hypertrophied after 28 days of fenoterol administration. Inflamation in intestinal glands was also observed.

Ileum

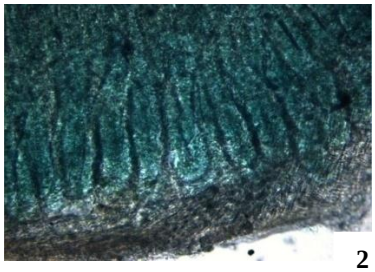
The ileum of normal mice revealed the presence of muscularis adventitia, muscularis externa, submucosa and mucosa stained with brown colour showing the presence of phospholipids. Lamina propria was stained with light black colour . Histochemical analysis showed that the goblet cells have acidic and neutral mucosubstances.

Villus hypertrophy was clearly noticed after 7 days of fenoterol administration. Increased number of goblet cells was appeared with almost negligible space in between the villi (Fig.19). Lamina propria was stained with light black colour. Epithelial disruption was also observed with vacuolization. Villi revealed patchy distribution of phospholipids after 14 days of drug treatment. Merging of the villi was noticed. There was no intervillar space noticed. Hypertrophy in the villus structure was also observed. Villi get abnormally elongated after 21 days of fenoterol treatment. Fusion of the villi was clearly observed (Fig.20). Phospholipids content get further decreased at this stage.

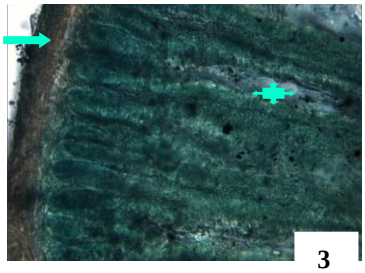
The structure of villus gets completely distorted after 28 days of fenoterol administration (Fig.21). Lamina propria was stained with light black colour . Epithelium disruption was also noticed. Intestinal absorptive cells at villus tips were ordinarily free of identifiable lipid droplets. Absorptive cells adjacent to crypts occasionally contained large lipid droplets at their bases.



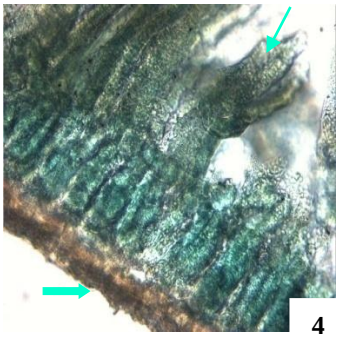
1



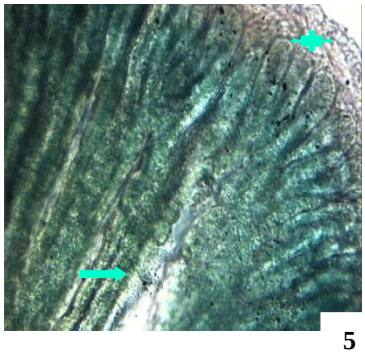
2



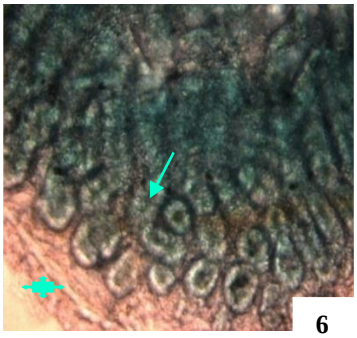
3



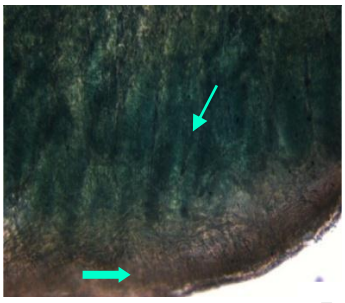
4



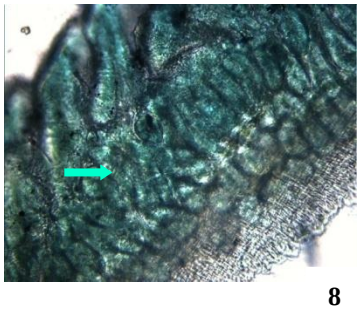
5



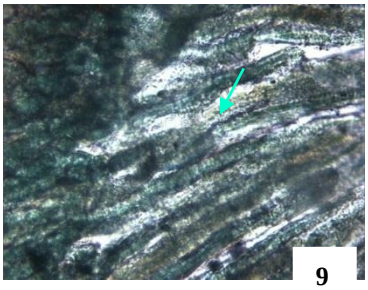
6



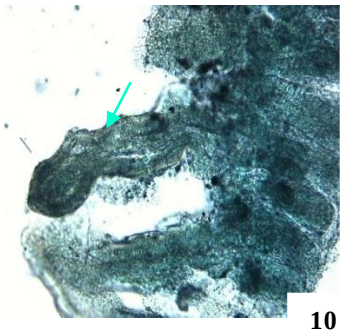
7

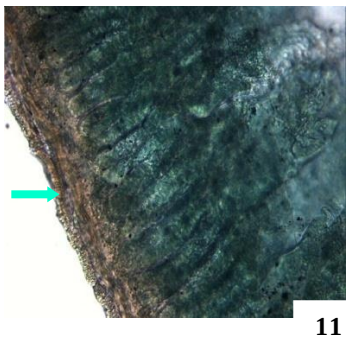


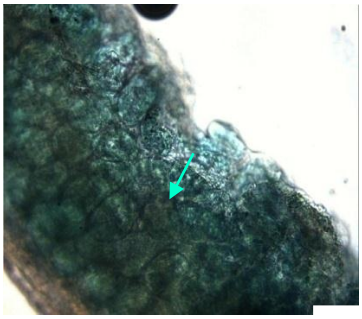
8

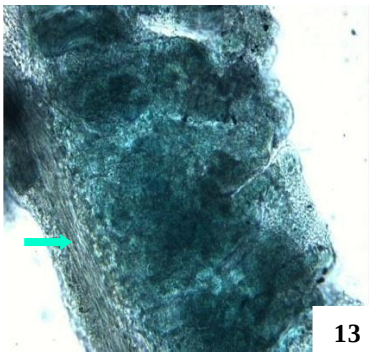


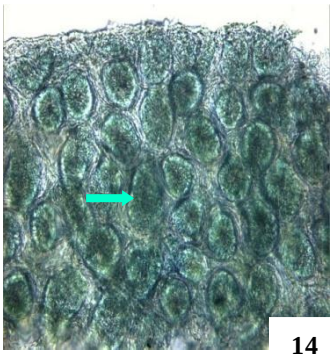
9

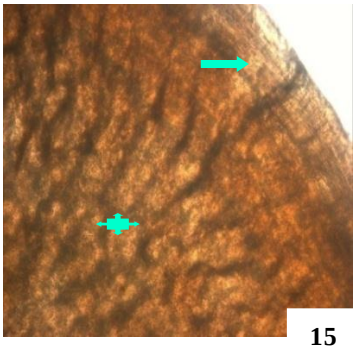

10


11

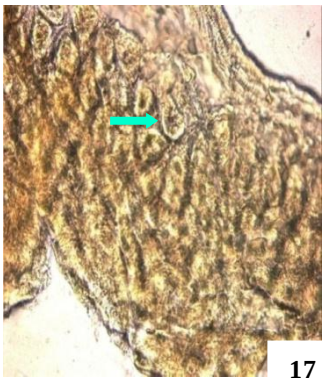

12

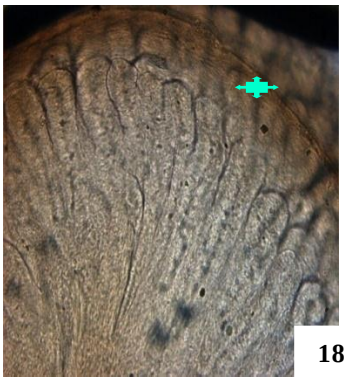

13

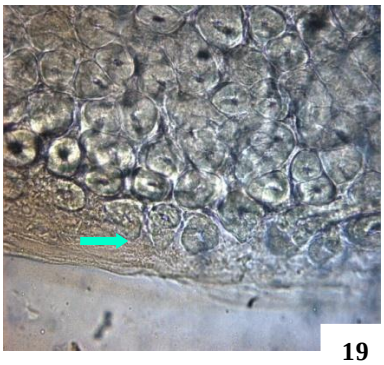

14


15

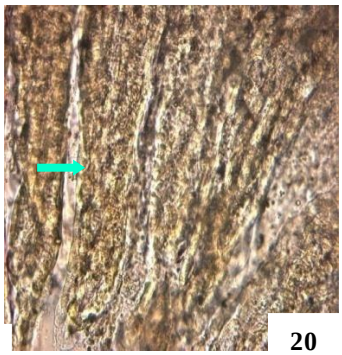

16


17

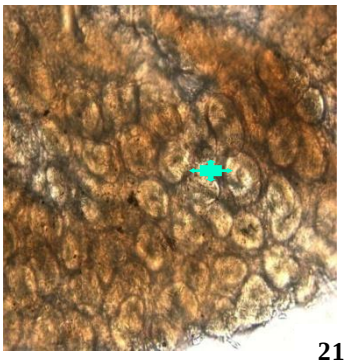

18



19



20



21

DISCUSSION

Phospholipids become increasingly important as formulation and as active ingredients. The present article summarizes particular features of commonly used phospholipids and their application spectrum within oral drug formulation and elucidates current strategies to improve bioavailability and disposition of orally administered drugs. Advantages of phospholipid formulations not only comprise enhanced bioavailability of drugs with low aqueous solubility or low membrane penetration potential, but also improvement or alteration of uptake and release of drugs, protection of sensitive active agents from degradation in the gastrointestinal tract, reduction of gastrointestinal side effects of non-steroidal anti-inflammatory drugs and even masking of bitter taste of orally applied drugs. Drug molecules must cross multiple cell membrane barriers to reach their site of action. The entire process, from drug adsorption to drug release within micelles, occurs on a time-scale of seconds, compatible with in vivo drug diffusion rates. Given the rate at which the reaction occurs, it is probable that this process is a significant mechanism for drug transport **(Magdelena *et al*., 2006).** Alterations in the acidic and neutral lipids were noticed in the drug treated mice. There was decrease in the lipid localization as a result of fenoterol treatment at 7 days stage. The mucosa of drug treated mice showed decrease in neutral lipid content as compared to normal duodenum mucosa. It was lightly stained with pink colour. The intestinal glands showed high acidic lipid accumulation. The neutral fats which were pink coloured due to metachromatic nature of the stain continue to increase with the increasing necrosis in the muscle fibres. After prolonged treatment of fenoterol it became clear that breakdown of acidic lipids takes place resulting in their metabolic transformations to neutral lipids. Such a metabolic transformation of lipids has also been observed by **Krishan (1982)** in

case of carcinomatous skeletal muscles and by **Sharma and Malhotra, (1991)** in chick skeletal muscle under stress conditions.

Since the neutral lipids are abundantly localized in the sarcoplasmic component exhibiting increasing necrotic changes after drug treatment, it is logical to expect a gradually increasing breakdown of acidic lipids in the sarcoplasm alongwith the phospholipids. The present histochemical evidence strongly supports the breakdown of phospholipid components after fenoterol treatment. Loss of phospholipids is further verified through the acid haematin test. The present study shows that phospholipids in the intact healthy fibers give a brownish colouration. Release of phospholipids into the interfibrillar and interfascicular spaces is predominantly observed. The villus get hypertrophied between 7- 28 days and phospholipids get decreased in the villi after drug treatment. The intestinal glands were stained with yellow colour which showed the decrease in phospholipid content.

Hypertrophy of the villus was observed after fenoterol treatment. The space between villus get decreased. The lamina propria was stained with light black colour. There was decrease in the phospholipid content in the villi as it was faintly stained with yellow colour. Merging of the villus was also observed. **Theologides (1979)** have discussed the implications of increased phospholipids and cholesterol content in the muscle but has not included any comment on lowered phospholipid levels in muscle fasiculi as revealed through present investigations. Studies have shown that an increase in phospholipids protects the mucosa from damage **(Litchenberger, 1983).**

Present study demonstrated for the first time that oral administration of fenoterol can enhance muscle apoptosis and hypertrophy. Although the improvements in regenerating fiber size and muscle function associated with a single dose of fenoterol were emphasized. The beta-adrenoceptor agonists have been used to relieve bronchoconstriction. Beta-agonists are based on adrenaline and early forms, such as isoprenaline, lacked bronchial selectivity and had unpleasant side effects. Modern beta-agonists are more selective for the β_2-adrenoceptors located in bronchial smooth muscle and have less cardiotoxicity. However, oral fenoterol administeration did have transient effects on some cardiovascular parameters, and these must be minimized before this form of treatment could be advocated for clinical application. The findings suggest

that fenoterol administration has therapeutic potential for conditions where muscle wasting and weakness are indicated.

REFERENCES

1. Thomas, A., Brasitus, S., Rajvir, D., Pradeep, K. and Bruce, M. **(1988)**. Cholesterol modulates alkaline phosphatase activity of rat intestinal microvillus membranes. *J. Biochem*. 263, (18): 8692 - 8697.
2. Dowhan, W. and Bogdanov, M. **(2009)**. Lipid-dependent membrane protein topogenesis. *Ann. Rev. Biochem.78*: 515 – 540.
3. Barenholz, Y. and Thompson, T.E. **(1980)**. Sphingomyelins in bilayers and biological membranes. *Biochim. Biophys. Acta*. 604: 129–158.
4. Dilley, R.J., Mc Geachie, J.K. and Prendergast, F. J. **(1987)**. A review of the proliferative behavior, morphology and phenotypes of vascular smooth muscle. *Atherosclerosis* 63: 99 - 107.
5. Owens, G.K. **(1989)**. Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am. J. Physiol*. 257: H1755- H1765.
6. Schwartz, S.M., Foy, L., Bowen - Pope, D.F. and Ross, R. **(1990)**. Derivation and properties of platelet derived growth factor - independent rat smooth muscle cells. *Am. J. Pathol*. 136: 1417-1428.
7. Thyberg, J., Hedin, U., Sfolund, M., Palmberg, L. and Bottger, B.A. **(1990)**. Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis* 10: 966 – 999.
8. Dzau, J.D. and Gibbons, G.H. **(1993)**. Vascular remodeling: mechanisms and implications. *J. pharmacol*. 21: 1- 5.
9. Lowell, L.M. **(1993)**. Remodeling of developing and mature arteries: Endothelin, smooth muscle . *J. Cardiovasc. Pharmacol*. 21: 11-17.
10. Schwartz, S.M. and Liaw, L. **(1993)**. Growth control and morphogenesis in the development and pathology of arteries. *J.Cardiovasc. Pharmacol.* 21: 31 – 49.

11. Dudenhausen, J.W., Hermer, M. and Rominger, K.L. **(1989)**. Mutterliche plasmakonzeentrationen being multipler oraler fenoterol behandling. *Geburtsh Frauenheilk* 49 : 234 - 236.
12. Nelson, H.S. **(1988)**. Beta - adrenergic therapy. *In: Allergy principles and practice.* (Eds. C.E. Middleton, R.C Reed and E.F Ellis). St. Louis: CV Mosby Co. pp. 647 - 664.
13. Jenne, J.W. and Tashkin, D.P. **(1993)**. Beta - adrenergic agonists. *In: Bronchial asthma-mechanisms and therapeutics.* (Eds. E.B. Weiss and M. Stein). Boston; Little, Brown and Co.1 pp. 746 – 83.
14. Cain, A.J. **(1947)**. *Q. J. Microsc. Sci* 88: 467. Cited from: *Histochemical Techniques* (Ed. J.D. Bancroft) Butterworths, London. (1975).
15. Baker, J.R. **(1946).** The histochemical recognition of lipine. *Q. J. Microsc. Sci*. 87: 441.
16. Magdalena, A., Baciu, L., Sarra, C., Sebai, L., Oscar ,L., Xavier, L., James, A., Clarke, L., Gemma, C., Shearman, L., Robert, V., Law, L., Richard, H., Templer, L., Christopheplisson Christine, A. and Parkerland A., G. **(2006)**. Degradative transport of cationic amphiphilic drugs across phospholipid bilayers. *Phil. Trans. R. Soc. A*. 364: 2597– 2614.
17. Krishan, K. **(1982)**. Pathological and metabolic aberrations in Carcinomatous skeletal muscle. Ph.D Thesis submitted to Himachal Pradesh University, Shimla.
18. Sharma S. and Malhotra R.K. **(1991).** Metabolic transformations of lipids in chick skeletal muscles under stress conditions. *J. Anim. Morphol. Physiol*. 38, 55-60.
19. Theologides, A. **(1979)**. Unfavourable signs in patients with chronic myelocytic leukaemia. *Ann. Intern. Med*. 76: 95.
20. Lichtenberger, L.M., Graziani, L.A., Dial, E.J., Butler, B.D. and Hills, B.A. **(1983)**. Role of surface - active phospholipids in gastric cytoprotection. *Science.*18; 219(4590):1327–1329.