CHAPTER – 4

REVERSED SERUM INSULIN AND GLUCOSE LEVELS AND GLYCOGENIC RESPONSE BUT POTENTIATED HEPATIC PROTEIN ANABOLIC RESPONSE FROM WEANING TO PUBERTAL AGE DUE TO NEONATAL MELATONIN ANTAGONISM.

INTRODUCTION:

Circadian secretion of melatonin from the pineal gland regulates a variety of physiological and neuroendocrine functions (Nowak and Zaurilska, 1998). Apart from controlling circadian changes, melatonin is also being increasingly implicated in the modulation of glycemic status and tissue carbohydrate metabolism (Ramachandran, 2002). Relationship between melatonin and the regulation of carbohydrate metabolism for long has been suggested both in humans (Alcozer *et al.*, 1956) and rodents (Milcu *et al.*, 1971). It has been shown in rats that pinealectomy decreases hepatic and muscle glycogenesis and increases blood pyruvate concentration (Milcu *et al.*, 1971).

An increase in blood sugar level subsequent to pinealectomy of rats has also been reported (Casaba and Barath, 1971). Influence of pinealectomy on several other physiological parameters related to carbohydrate metabolism has also been recorded (Diaz and Blazquez,

1986). Conversely, infusion of pineal extracts has been noted to result in hypoglycemia and increased glucose tolerance and hepatic and muscle glycogenesis after glucose loading (Milcu *et al.*, 1971). Studies on isolated adipocytes from rat epididymal fat have shown enhanced cell sensitivity to insulin. A generalized role for melatonin in carbohydrate homeostasis and dose, time and species specific differential responses have also been brought out (Ramachandran, 2002). The multibiologic effects of melatonin have been related to its role as a powerful antioxidant (Carnerio and Reiter, 1998) as well as its interactions with the specific receptors (Witt-Enderby and Li, 2000). Biochemically, usage of selective agonist or antagonist is not only important in identifying melatonin receptor subtypes but can also be used as an important tool for identifying specific physiological functions of melatonin, an added advantage over the generalized effects of pinealectomy. Luzindole has been identified as a most specific antagonist for the MT_2 subtype of receptors more than for MT_1 (Dawson and Van Den Heuvel, 1998; Zhou *et al.*, 2003).

A previous study tended to identify the immediate consequences of chronic melatonin antagonism during the entire pre-weaning period on parameters of carbohydrate metabolism on the weaning day (Chapter-I). Significant alterations in glycogen metabolising enzymes and on the status of glycogenesis, glycemia and insulinemia were recorded in the above study due to luzindole induced melatonin antagonism. Further altered insulin sensitivity of liver and muscle was also noted (Chapter-2). In continuation of the above studies, the present investigation has

been carried out to study specifically the long term effects of chronic melatonin antagonism in the pre-weaning period on parameters of carbohydrate metabolism at the pubertal age.

MATERIAL AND METHODS: See page numbers 18-38.

RESULTS:

- **Body and organ weights:** The luzindole treated animals showed marginal although non significant increase in body weight as compared to controls. Whereas, the relative weights of pancreas, and adrenals decreased significantly, those of spleen, testes and kidney increased significantly in the experimental animals. The relative weight of liver increased marginally although non significant in the experimental animals (Figure and Table; 4.1, 4.4, 4.5).
- **Serum glucose and insulin levels:** Whereas the serum glucose level decreased significantly, the serum insulin level showed a significant increase in the luzindole treated rats (Figure and Table; 4.6).
- **Hepatic glycogen content and activities of glycogen synthetase, glycogen phosphorylase and glcose-6-phosphatase:** The hepatic glycogen content remained unaltered while the, activities of glycogen phosphorylase and glucose-6-phosphatase were elevated, while the activity of glycogen synthetase decreased significantly in the experimental rats (Figure and Table; 4.7, 4.8, 4.9, 4.10).

Figure 4.1: Body weight and absolute weight of liver of pubertal rats on 45th day subjected to neonatal luzindole treatment:

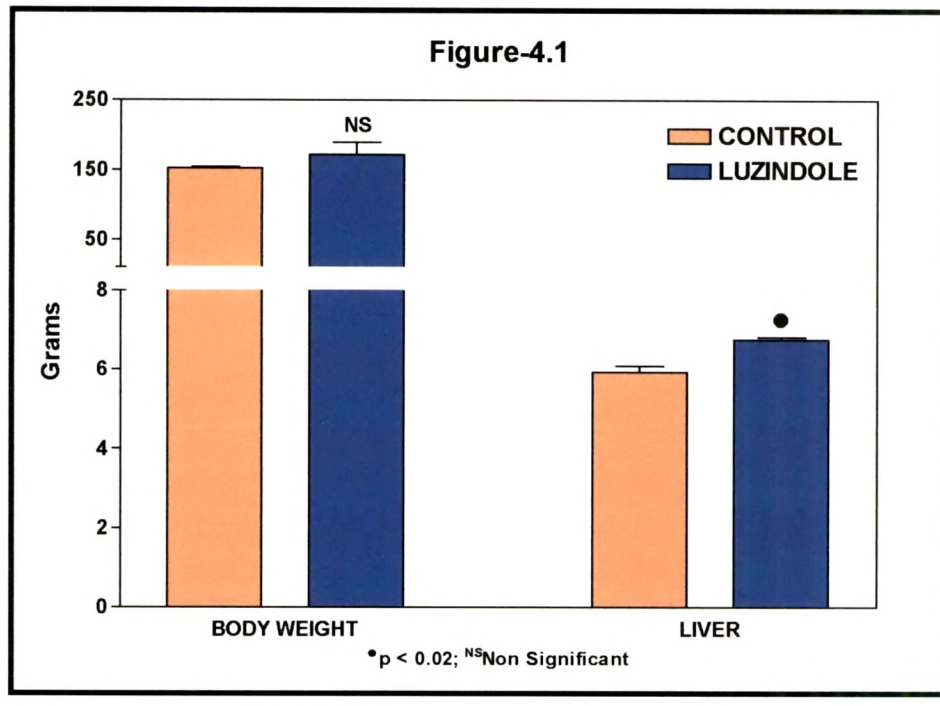


Table 4.1: Body weight and absolute weight of liver of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	BODY WEIGHT	LIVER
CONTROL	152.50 ±2.3	5.94 ±0.15
LUZINDOLE	172.5[NS] ±17.54	6.76[•] ±0.059

Values are expressed as mean ± SEM, [NS]Non Significant; [•] p < 0.02

Figure 4.2: Absolute weight of pancreas, spleen and adrenal of pubertal rats on 45th day subjected to neonatal luzindole treatment:

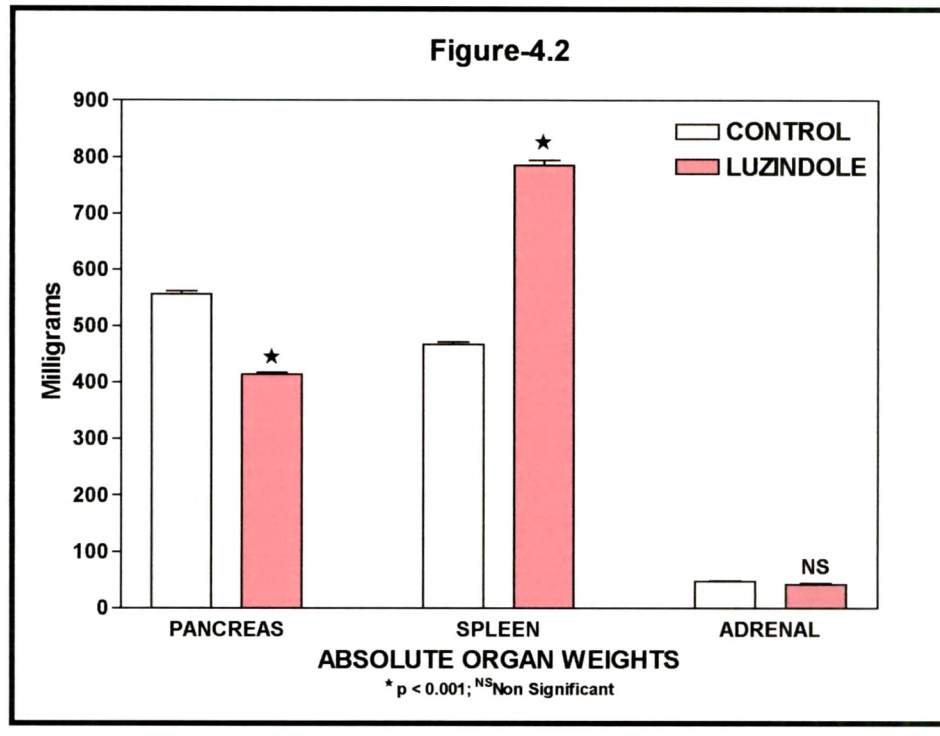


Table 4.2: Absolute weight of pancreas, spleen and adrenal of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	PANCREAS	SPLEEN	ADRENAL
CONTROL	557.00 ±2.50	467.50 ±4.51	47.5 ±1.50
LUZINDOLE	414.00* ±4.00	785.50* ±8.52	42.00NS ±2.00

Values are expressed as mean ± SEM, *p < 0.001; NS Non Significant

Figure 4.3: Absolute weight of testes and kidney of pubertal rats on 45th day subjected to neonatal luzindole treatment:

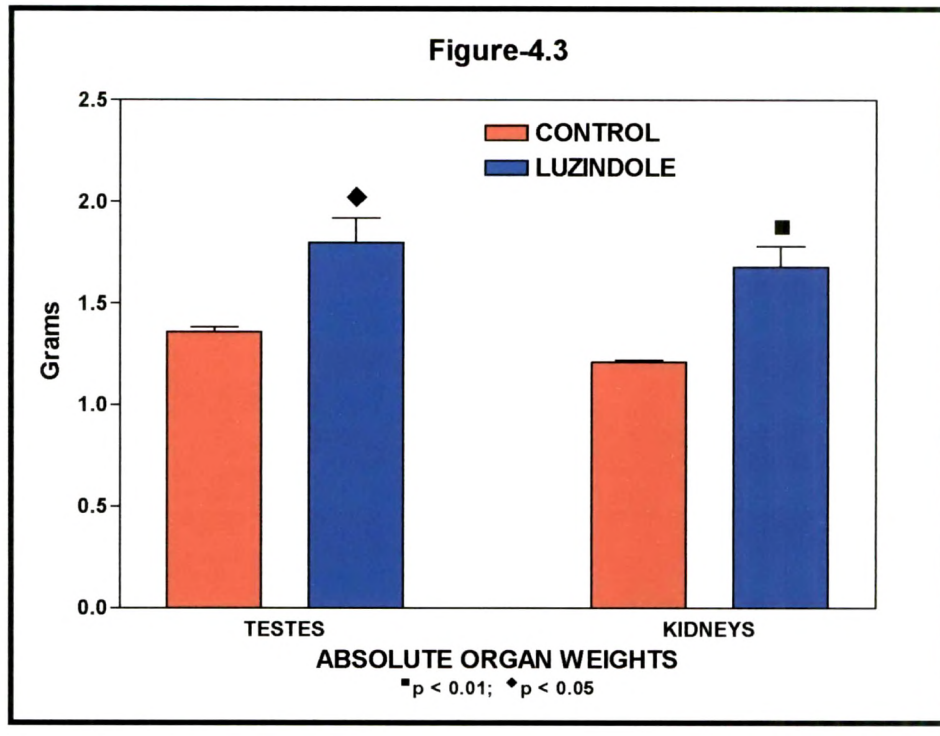


Table 4.3: Absolute weight of testes and kidney of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	CONTROL	LUZINDOLE
TESTES	1.36 ±0.024	1.80◆ ±0.12
KIDNEYS	1.21 ±0.0099	1.68■ ±0.10

Values are expressed as mean ± SEM, ■ p < 0.01; ◆p < 0.05

Figure 4.4: Relative weight of pancreas, spleen and adrenals of pubertal rats on 45th day subjected to neonatal luzindole treatment:

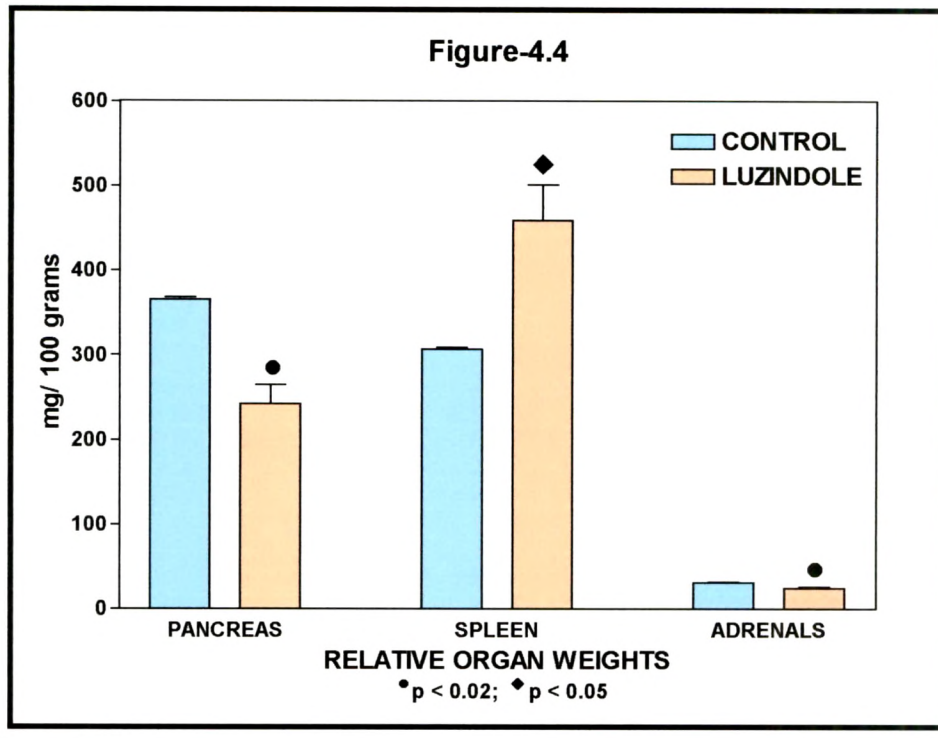


Table 4.4: Relative weight of pancreas, spleen and adrenals of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	PANCREAS	SPLEEN	ADRENALS
CONTROL	365.29 ±2.71	306.58 ±2.07	31.13 0.47
LUZINDOLE	242.25• ±22.31	459.59◆ ±41.82	24.47• ±1.32

Values are expressed as mean ± SEM, • p < 0.02; ◆p < 0.05

Figure 4.5: Relative weight of liver, testes and kidney of pubertal rats on 45th day subjected to neonatal luzindole treatment:

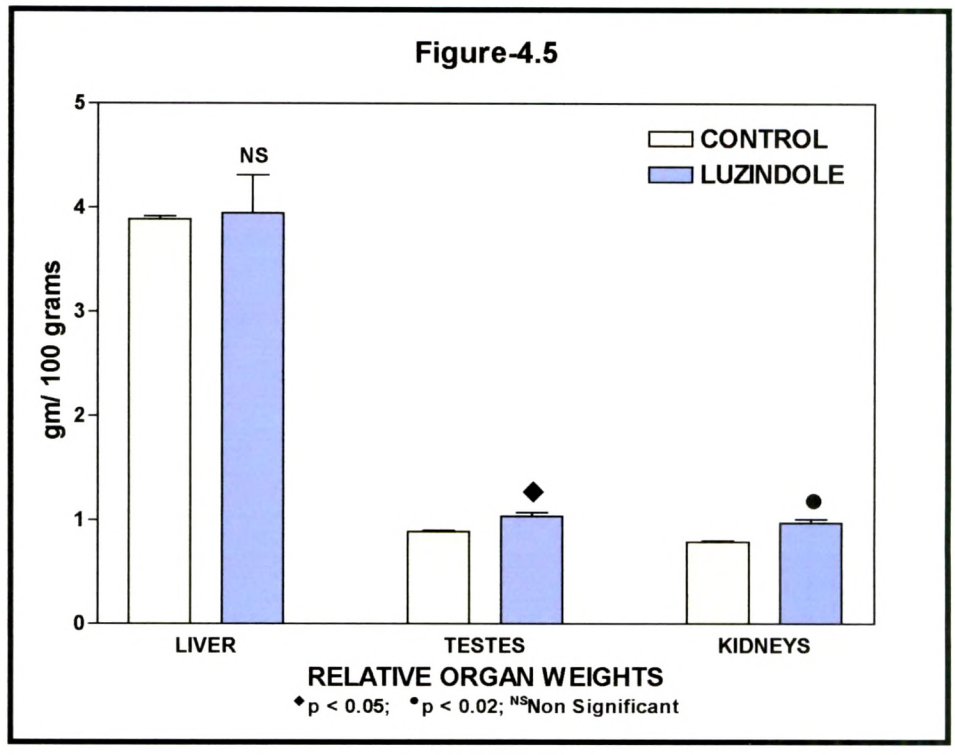


Table 4.5: Relative weight of liver, testes and kidney of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	LIVER	TESTES	KIDNEYS
CONTROL	3.89 ±0.03	0.89 ±0.01	0.79 0.0099
LUZINDOLE	3.95[NS] ±0.36	1.04[◆] ±0.034	0.97[•] ±0.034

Values are expressed as mean ± SEM, ◆ p < 0.05; • p < 0.02; NS Non Significant

Figure 4.6: Serum insulin and glucose levels of pubertal rats on 45th day subjected to neonatal luzindole treatment:

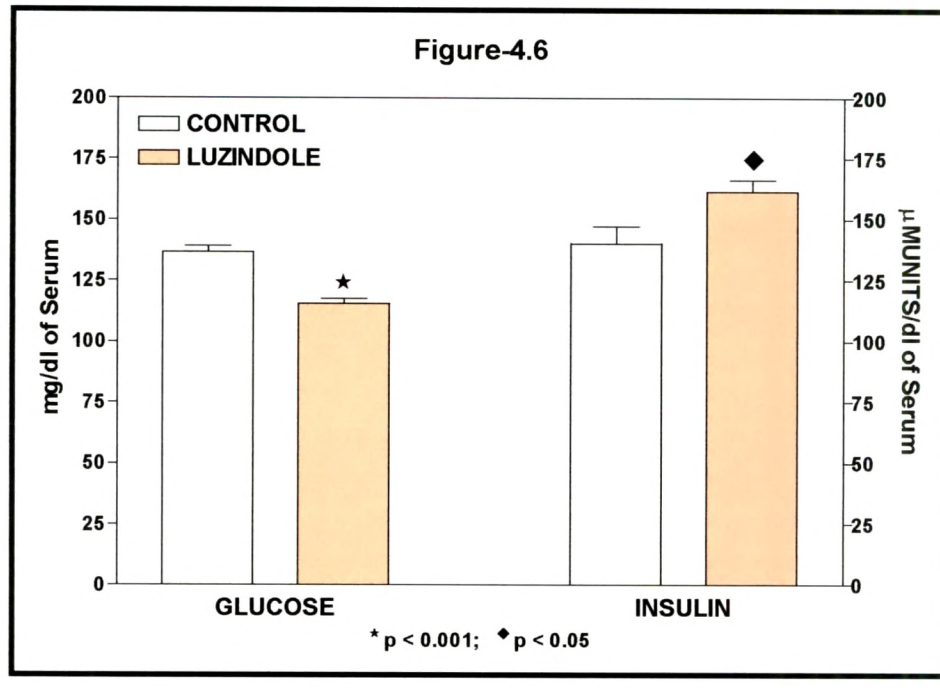


Table 4.6: Serum insulin and glucose levels of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	INSULIN	GLUCOSE
CONTROL	140.27 ±7.0682	136.72 ±2.379
LUZINDOLE	161.65◆ ±4.8055	115.55* ±2.0411

Values are expressed as mean ± SEM, * p < 0.001; ◆p < 0.05

Figure 4.7: Activity of glycogen phosphorylase in liver and muscle of pubertal rats on 45th day subjected to neonatal luzindole treatment:

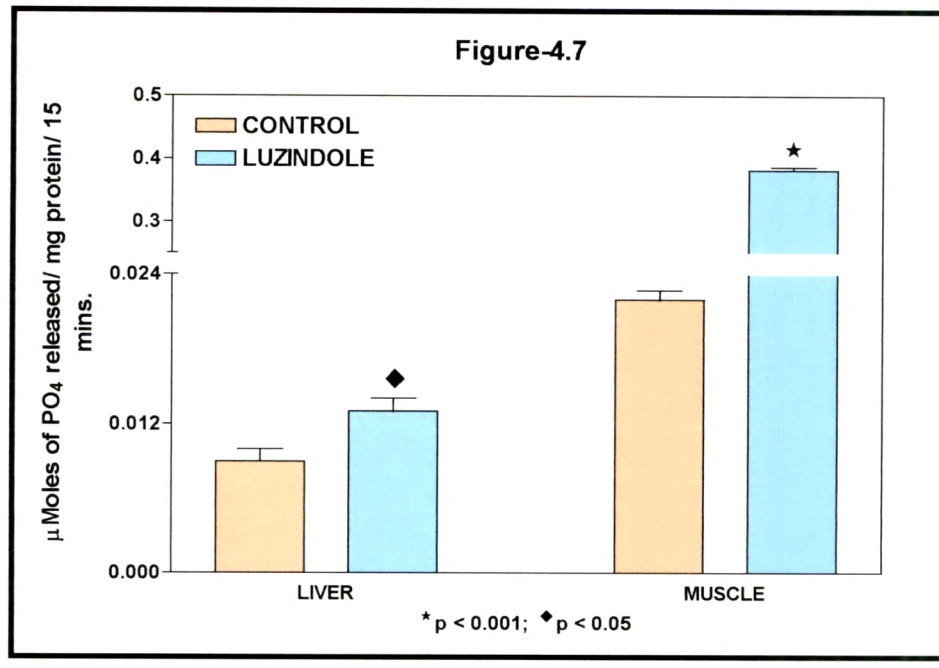


Table 4.7: Activity of glycogen phosphorylase in liver and muscle of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	0.009 ±0.00095	0.022 ±0.00075
LUZINDOLE	0.013* ±0.00105	0.38◆ ±0.0048

Values are expressed as mean ± SEM, * p < 0.001; ◆p < 0.05

Figure 4.8: Glucose-6-phosphatase activity in the liver of pubertal rats on 45th day subjected to neonatal luzindole treatment:

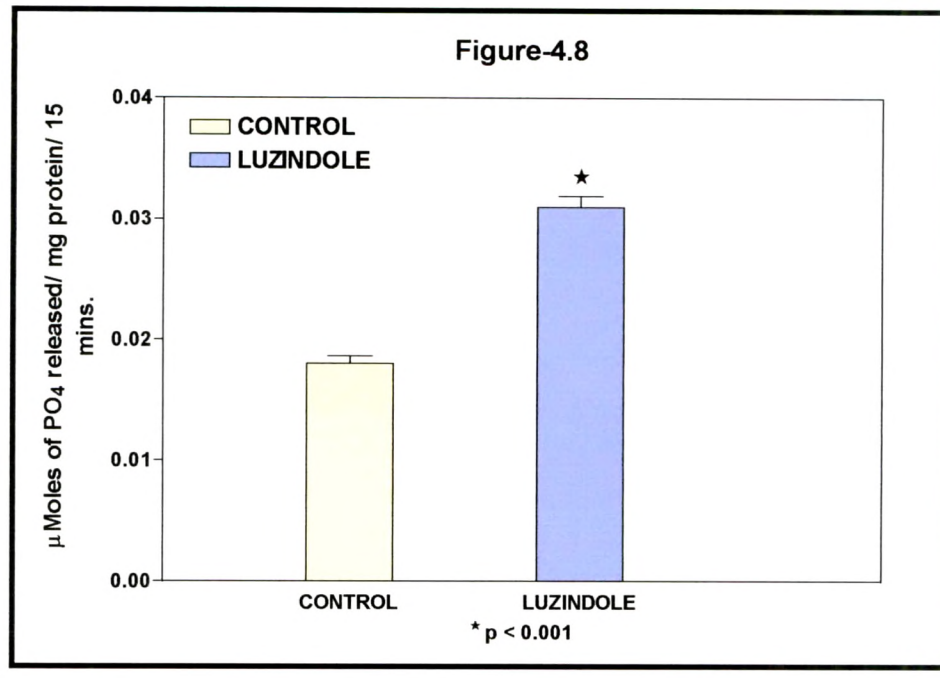


Table 4.8: Glucose-6-phosphatase activity in the liver of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	CONTROL	LUZINDOLE
GLUCOSE-6-PHOPHATASE	0.018 ±0.0006	0.031* ±0.0009

Values are expressed as mean ± SEM, *p < 0.001

Figure 4.9: Glycogen synthetase activity in liver and muscle of pubertal rats on 45th day subjected to neonatal luzindole treatment:

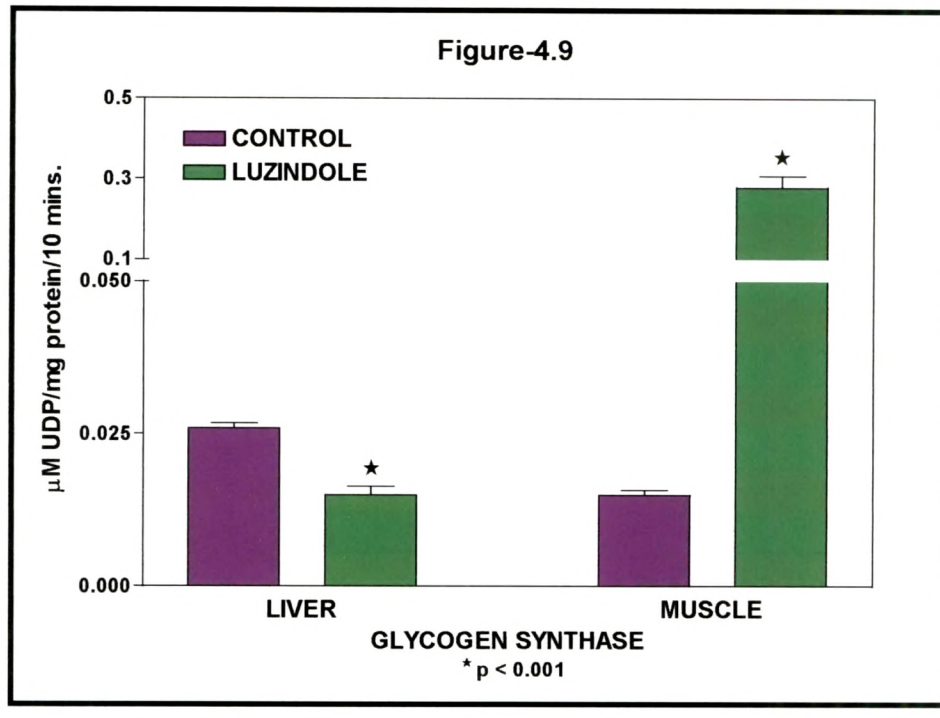


Table 4.9: Glycogen synthetase activity in liver and muscle of pubertal rats on 45th day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	0.026 ±0.00075	0.015 ±0.00085
LUZINDOLE	0.015* ±0.0014	0.280* ±0.02865

Values are expressed as mean ± SEM, * p < 0.001

Figure 4.10: Hepatic and muscle glycogen content of the pubertal rats on 45th day subjected to neonatal luzindole treatment:

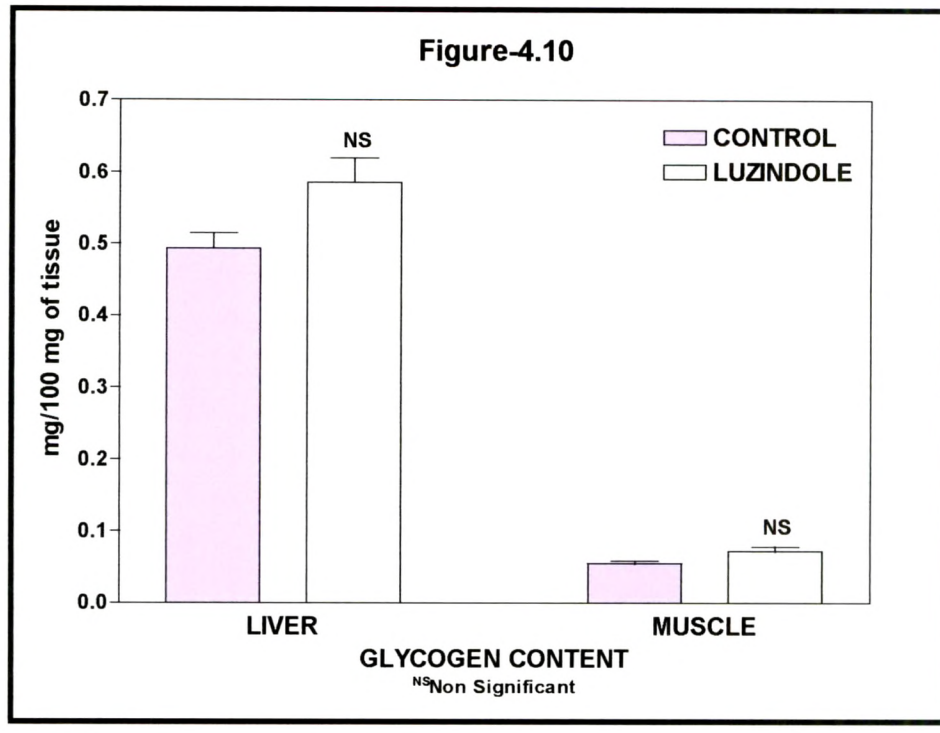


Table 4.10: Hepatic and muscle glycogen content of the pubertal rats on 45th day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	0.4933 ±0.0275	0.0547 ±0.00395
LUZINDOLE	0.5857[NS] ±0.03365	0.0719[NS] ±0.0069

Values are expressed as mean ± SEM, [NS] Non Significant

Figure 4.11: Hepatic and muscle protein content of the pubertal rats on 45th day subjected to neonatal luzindole treatment:

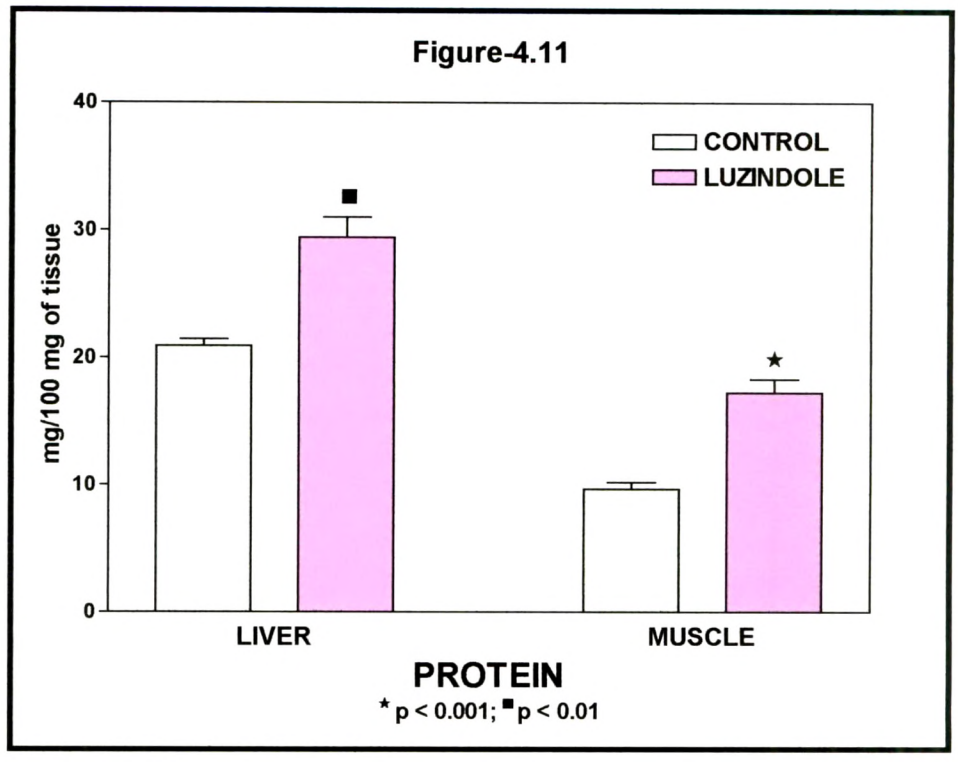


Table 4.11: Hepatic and muscle protein content of the pubertal rats on 45th day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	20.92 ±0.5416	9.66 ±0.5385
LUZINDOLE	29.43* ±1.614	17.29■ ±1.058

Values are expressed as mean ± SEM, * p < 0.001; ■ p < 0.01

PLATE – 6

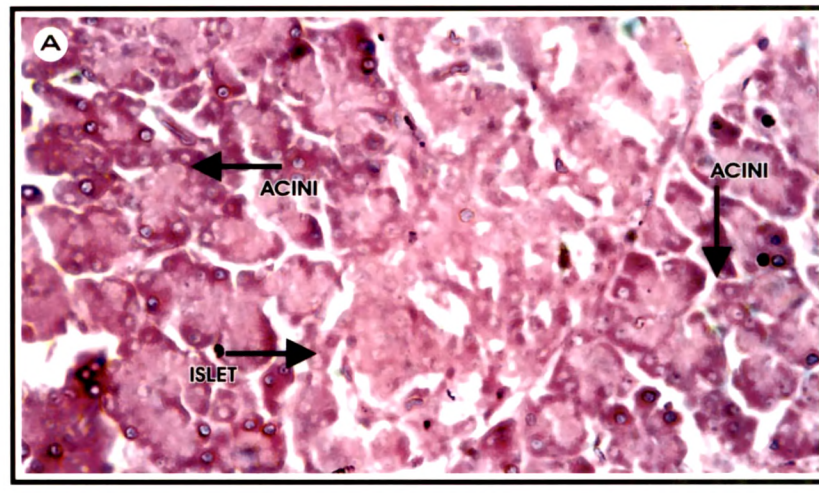
Photomicrographs of sections of pancreas – 450 X

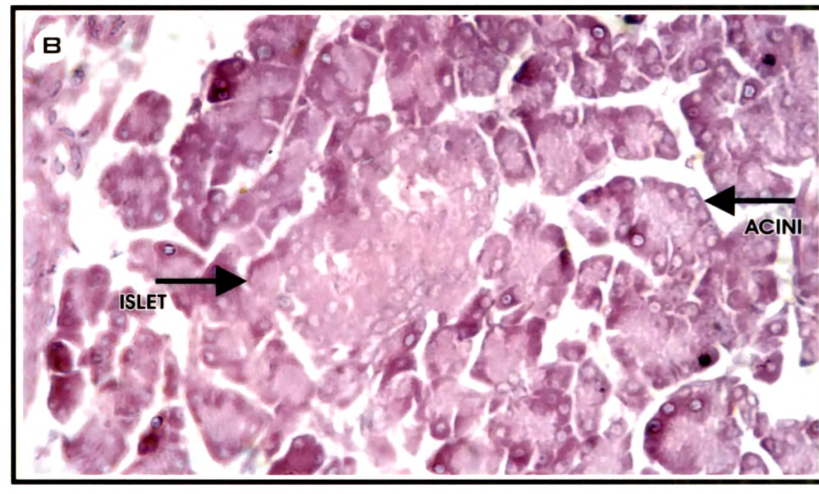
FIGURE (A): Transverse section of the pancreas of male luzindole treated rats on the 45th day showing islet and pancreatic acini. There is an increase in the islet size and islet cell number, with an increased B:A cell ratio.

FIGURE (B): Transverse section of the pancreas of male control pubertal (45th day) rats showing islet and pancreatic acini. Note the centrally distributed A cells, peripherally distributed B cells.

FIGURE (C): Transverse section of the pancreas of male luzindole treated rats on the 45th day showing islet and pancreatic acini. There is an increase in the islet size and islet cell number, with an increased B:A cell ratio.

PLATE - 6





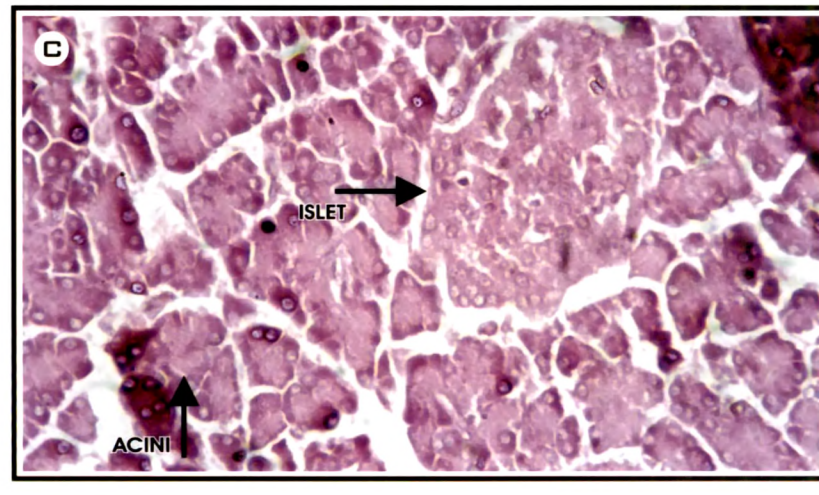


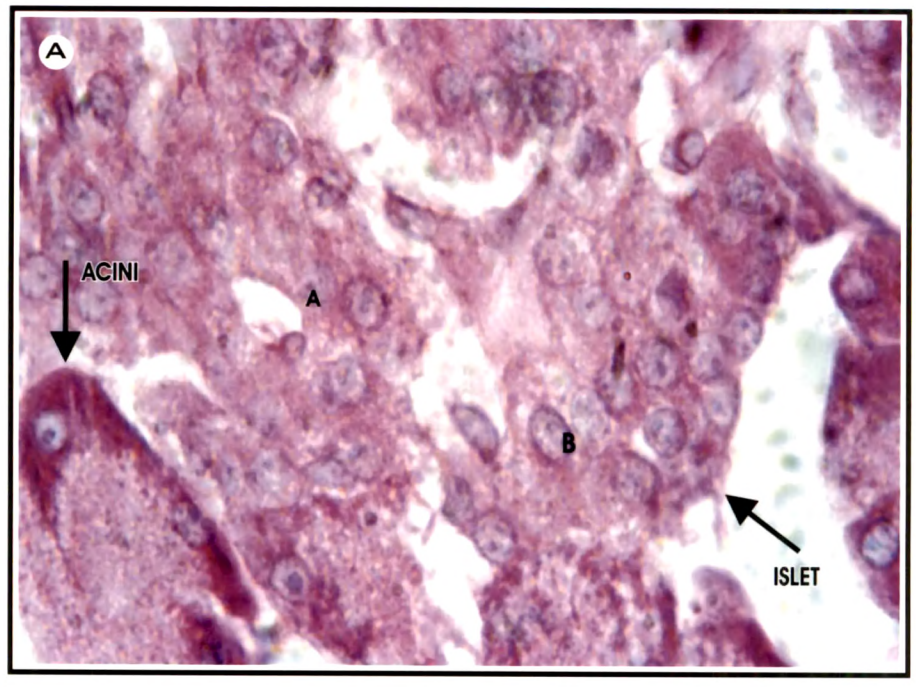
PLATE – 7

Photomicrographs of sections of pancreas – 1000 X

FIGURE (A): Transverse section of the pancreas of male control pubertal (45^{th} day) rats showing islet and pancreatic acini. Note the centrally distributed A cells and peripherally distributed B cells.

FIGURE (B): Transverse section of the pancreas of male luzindole treated rats on the 45^{th} day showing islet and pancreatic acini. The number of B cells in the islet is increased as compared to the A cells.

PLATE - 7



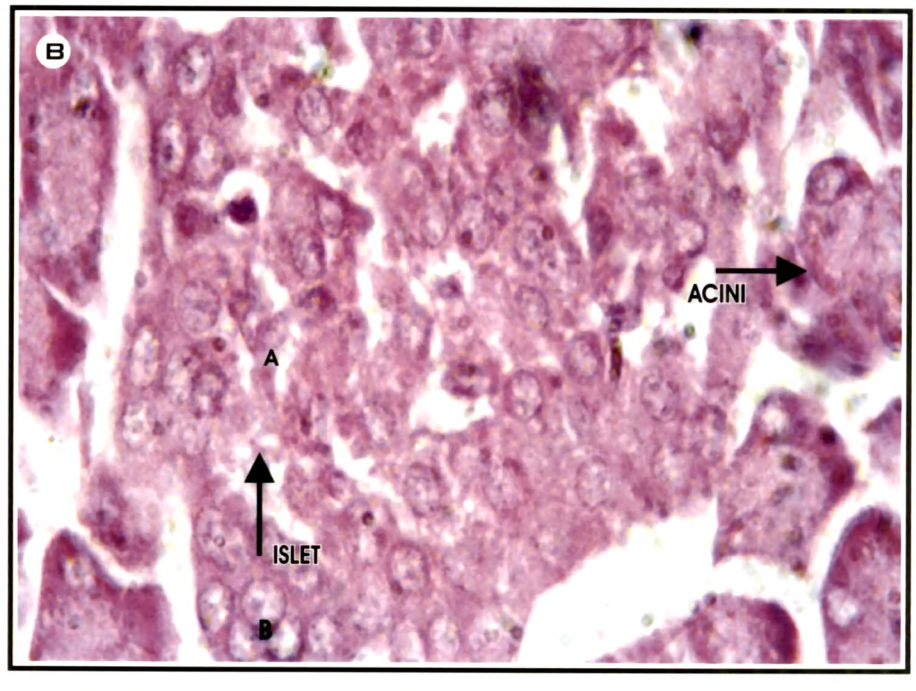


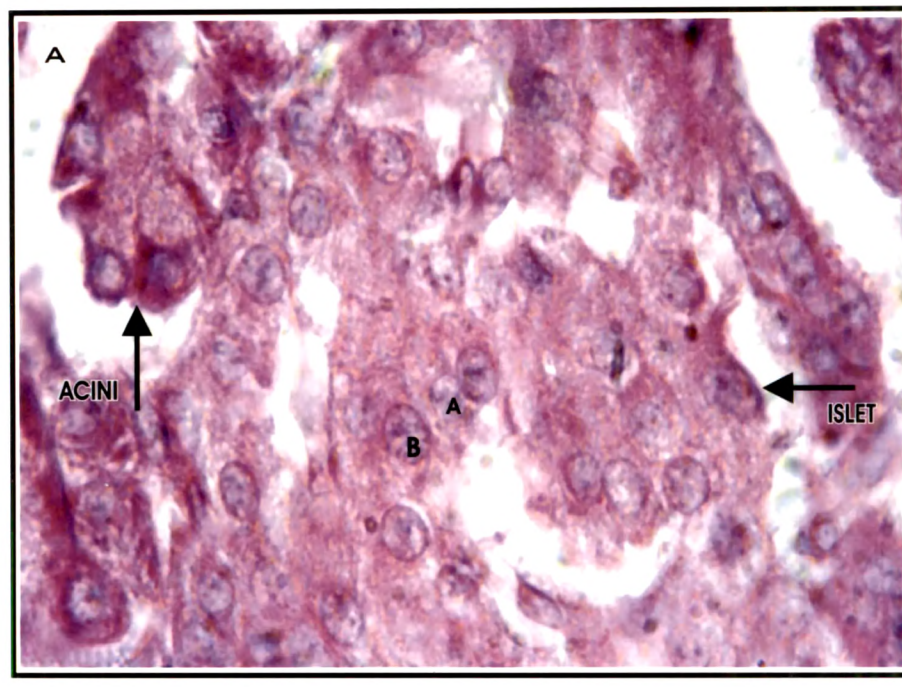
PLATE – 8

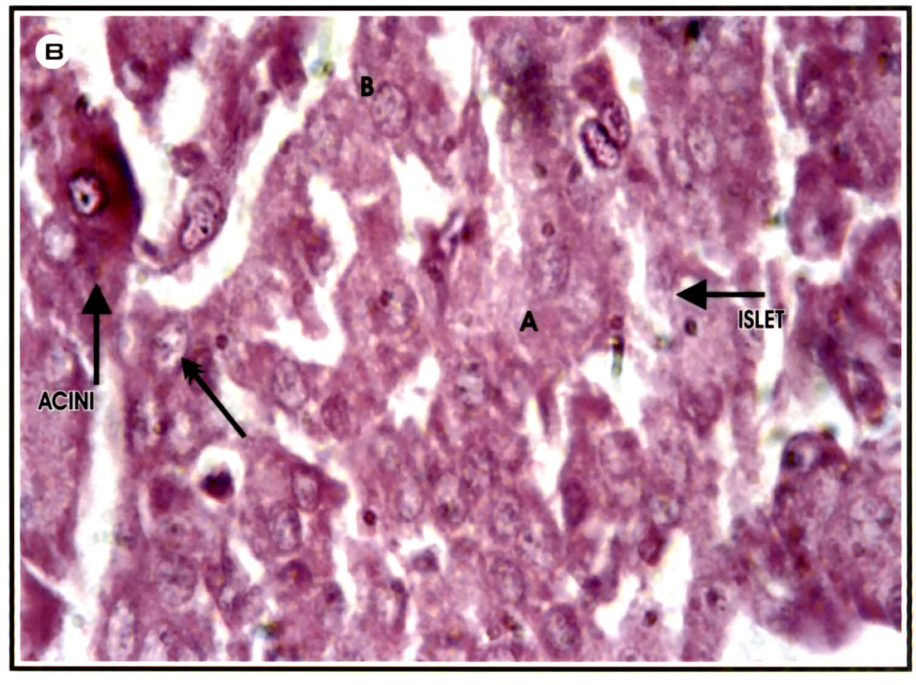
Photomicrographs of sections of pancreas – 1000 X

FIGURE (A): Transverse section of the pancreas of male control pubertal (45^{th} day) rats showing islet and pancreatic acini. Note the centrally distributed A cells and peripherally distributed B cells.

FIGURE (B): Transverse section of the pancreas of male luzindole treated rats on the 45^{th} day showing islet and pancreatic acini. The number of B cells in the islet is increased as compared to the A cells. Note the transdifferentiation (double headed arrow) of the acinar cells into the islet cells.

PLATE - 8





- **Muscle glycogen content and activities of glycogen synthetase and glycogen phosphorylase:** The luzindole treated animals showed a significant increase in the activities of glycogen synthetase and glycogen phosphorylase in the muscle as compared to that of the control animals while the, glycogen content in the muscle of the experimental animals showed no significant alteration (Figure and Table; 4.7, 4.9, 4.10).
- **Hepatic and muscle protein content:** The hepatic and muscle protein contents increased significantly in the luzindole treated rats. The increase was more pronounced in the muscle tissue (Figure and Table; 4.11).
- **Histological observations:** The islets of luzindole treated rats appear to be larger in size with higher number of B and A cells and a higher B:A ratio. Transdifferentiation of acinar cells into islet cells seen pronouncedly at 22nd day of luzindole treated rats is found to be still persistent though to a lesser degree (Plate; 6, 7, 8).

DISCUSSION:

Previous study on melatonin antagonism during neonatal period had shown significantly increased hepatic and muscle glycogen contents with decreased glycogen synthetase: glycogen phosphorylase activity ratio and hypoglycemia coupled with hyperinsulinemia. The increased tissue glycogen content despite the decreased synthetase: phosphorylase activity ratio had been accredited to the significant increase in serum insulin level tending to push the tissues to glycogen

storage (Chapter-I). The present study is to essentially evaluate the long term effect of previous melatonin antagonism in the neonatal period on pubertal carbohydrate homeostasis as compared to the immediate post blockage effect in the weaning period. It is clear that both hepatic and muscle glycogen contents have shown significant decrease in luzindole treated rats as compared to the significantly increased contents in the control rats. Corresponding to the increase in tissue glycogen contents in control rats there is increased synthetase: phosphorylase activity ratio. Hyperinsulinemia paralleled by hypoglycemia also support the observed glycogenic effect. The recorded hyperinsulinemia is well borne out by the higher B:A cell ratio in the islets of luzindole treated rats (Plate; 6, 7, 8). It is presumed that the increased serum insulin level and increased insulin sensitivity are the major influence on the observed 945% and 131% increase in hepatic and muscle glycogen contents respectively in the control and luzindole treated rats. In contrast, the luzindole rats showed decreased hepatic glycogen content despite higher GS: GP ratio and increased insulin level. Though the serum glucose level is decreased the tissue glycogen contents (liver and muscle) are also decreased despite increased GS: GP ratio in the former or unaltered in latter, suggesting decreased insulin sensitivity. Increased glucose-6-phosphatase activity in luzindole rats compared to decreased activity in the control rats also correlate well with the decreased glycogenic effect in the former and increased effect in the latter. Obviously from weaning to puberty, there is increasing tissue glycogen content and hyperinsulinemia, with

increasing tissue insulin sensitivity. However, melatonin antagonism in the neonatal period results in reverse set of changes suggesting persisting insulin resistance. In general there is increasing protein anabolic influence from weaning to puberty which is more markedly manifested in the luzindole rats. Though there is no obesity, luzindole rats show slightly higher body weight and significantly increased relative weight of spleen, kidney, testes and adrenal with no change in the weight of pancreas and liver. This may suggest that melatonin has a growth regulatory influence on spleen, kidney, testes and adrenal. In keeping with the puberty observed decreased insulin sensitivity and glycogenic influence are the reports of impaired glucose homeostasis in pinealectomized rats marked by diminished glucose tolerance, insulin resistance, decreased hepatic and muscle glycogenesis and increase in the blood pyruvate concentration (Csaba and Barath, 1971; Milcu *et al.*, 1971; Mellado *et al.*, 1986; Diaz and Blazquez, 1986). Additionally, pinealectomy has been shown to decrease insulin response and a fall in GLUT-4 content in adipose and muscle tissue (Lima *et al.*, 1998; Zanquetta *et al.*, 2003). Though the studies are on pinealectomized rats beyond the pubertal age or, at the pubertal age, and involve complete absence of pineal, the present study is specifically on melatonin antagonism in the neonatal age and the effects manifested in the post antagonism period quite sometime after the withdrawal of the antagonist. Clearly, the present study tends to provide information on direct and specific effect of absence of melatonin action and also in the continued and prolonged action on a

time scale extending to nearly a month. Since the adrenal glands of luzindole rats have shown a significant increase it is likely that there is increased corticosterone titre in these animals which could again contribute to insulin resistance/insensitivity. This is supported by previous studies showing increased blood corticosterone concentration due to pinealectomy (Whichlow *et al.*, 1974), modulatory influence of melatonin on cell response to glucocorticoid (Aoyama *et al.*, 1988; Mori *et al.*, 1984) and, the suggestion in the above studies that lack of the pineal gland can cause hyperfunctioning of the adrenocortical physiology, which can lead to insulin resistance (Lima *et al.*, 1998).

In conclusion it can be summarized that neonatal blockage of melatonin action results in reduced tissue insulin sensitivity which persists for quite sometime extending into the pubertal period. This is manifested in the form of decreased glycogenic effect and probably potentiated protein anabolic influence in association with the pubertal increase in testosterone.

SUMMARY:

A previous study tended to identify the immediate consequences of chronic melatonin antagonism during the entire pre-weaning period on parameters of carbohydrate metabolism on the weaning day. In continuation of the above studies, the present investigation has been carried out to decipher specifically, the long term effects of chronic melatonin antagonism in the pre-weaning period on parameters of carbohydrate metabolism at the pubertal age. To this end, rat neonates have been treated with Luzindole (An MT_2 receptor blocker)

(400 µg/Kg body weight) intra peritoneally from day 1 to day 21 and assessed on the 45th day. Though the body weight of luzindole treated animals showed no significant alteration as compared to controls, the relative weight of pancreas and adrenals decreased significantly while, that of spleen, testes and kidney increased. Whereas the serum glucose level decreased significantly, the serum insulin level showed a significant increase in the experimental rats. The hepatic and muscle glycogen content of the luzindole treated rats showed no significant alteration as compared to controls. The activity of glycogen phosphorylase increased significantly in the liver and muscle of experimental rats while, the glycogen synthetase activity decreased in the liver. The muscle glycogen synthetase increased significantly in the luzindole treated rats but the glucose-6-phosphatase activity in the liver also increased significantly experimental rats. The islets of luzindole treated rats appear to be larger in size with higher number of B and A cells and a higher B:A cell ratio. Transdifferentiation of acinar cells into islet cells seen prominently at 22 days of luzindole treated rats is found to be still persistent though to a lesser degree. Overall, the present observations indicate that the neonatal blockage of melatonin action can result in reduced tissue sensitivity to insulin? Which, persists for quite sometime extending into the pubertal period. This is manifested in the form of decreased glycogenic effect and probably potentiated protein anabolic influence in association with the pubertal increase in testosterone.