CHAPTER – 1

NEONATAL MELATONIN ANTAGONISM IN THE PRE-WEANING PERIOD INDUCES HYPERINSULINEMIA, DECREASES INSULIN SENSITIVITY AND ALTERS CARBOHYDRATE HOMEOSTASIS.

INTRODUCTION:

Though synthesized in many peripheral organs, melatonin produced by the pineal gland remains principally the evocator of circadian and seasonal behavior and physiology (Reiter, 1991; Pang *et al.*, 1992; Reiter, 1993). The nocturnal release of melatonin times the various mammalian circadian rhythms (Arendt, 1995) and regulates reproductive changes in response to changing photoperiod in seasonally breeding mammals (Bartness *et al.*, 1993) and birds (Ramachandran and Patel, 1986; Patel *et al.*, 1985). The pineal gland, by synthesizing and releasing melatonin, plays an important role in the interface between the environment and rhythmic physiological activities of the vertebrate body. The neuroendocrine system that regulates the pineal gland times the production and secretion of melatonin to the dark phase of the diurnal environmental lighting cycle. Thus the daily production of melatonin in relation to environmental lighting cycles helps transmit daily and seasonal timing cues to the internal *milieu*. A

wide range of physiological functions is being regulated and modulated by the internally generated melatonin signal (Amstrong, 1989; Carneiro *et al.*, 1991; Cipolla-Neto *et al.*, 1991). An indication for the role of pineal gland and melatonin in the regulation of carbohydrate metabolism had come quite early from studies on humans and rodents (Alcozer *et al.*, 1956; Milcu *et al.*, 1971). It has been demonstrated that pinealectomized rats show decreased hepatic and muscle glycogenesis and an increase in blood pyruvate concentration (Milcu *et al.*, 1971; Mellado *et al*,. 1986). Further, pinealectomy has been shown to increase blood sugar levels in normal as well as alloxan treated rats (Csaba and Barath, 1971). Effects of pinealectomy on many other physiological parameters involved in carbohydrate metabolism have also been demonstrated (Diaz and Blazquez, 1986). In recent times a relationship between melatonin and regulation of carbohydrate metabolism is under increasing scrutiny (Van Cauter *et al.*, 1989, 1991; Lima *et al.*, 1998; Peschke and Peschke, 1998). Enhanced adipocyte sensitivity to insulin induced by melatonin has also been demonstrated (Lima *et al.*, 1994). As a corollary, pinealectomy has been shown to decrease insulin response and manifest a fall in GLUT-4 content in adipose and muscle tissues (Lima *et al.*, 1998). It has also been shown that melatonin suppresses insulin secretion under several experimental conditions (Feldman and Lebovitz, 1972; Peschke and Peschke, 1998; La Fleur, 2001).

A parallel study being conducted in the laboratory has shown neonatal hypermelatonemia to decrease serum insulin, increase insulin

sensitivity and alter carbohydrate homeostasis (Jani, 2004). The above study clearly shows a greater impact of altered melatonin status in the neonatal period. The present study in this backdrop has been designed to test the effect of blockage of melatonin action on serum insulin and glucose levels and hepatic and muscle carbohydrate homeostasis. To this end rat neonates have been treated with luzindole (An MT_2 receptor antagonist) from day 1 to day 21 and the serum insulin and glucose status and hepatic and muscle glycogen and protein contents and enzymes involved in carbohydrate metabolism have been assessed on the 22^{nd} day.

MATERIAL AND METHODS: See page numbers 18-38.

RESULTS:

- **Body and Organ weights:** There is a significant decrease in the body weight of luzindole treated animals. The relative weights of pancreas and kidneys showed no significant alterations while, those of liver, spleen and adrenals showed a significant increase. The relative weight of testes showed a significant decrease as compared to controls (Fig. and Tab. 1.1, 1.3 and 1.4)
- **Serum glucose and insulin levels:** Serum glucose level showed a significant decrease while serum insulin level increased significantly in the experimental neonates as compared to controls (Fig. and Tab. 1.5).

Figure 1.1: Body and absolute weight of liver of weaning rats on 22nd day subjected to neonatal luzindole treatment:

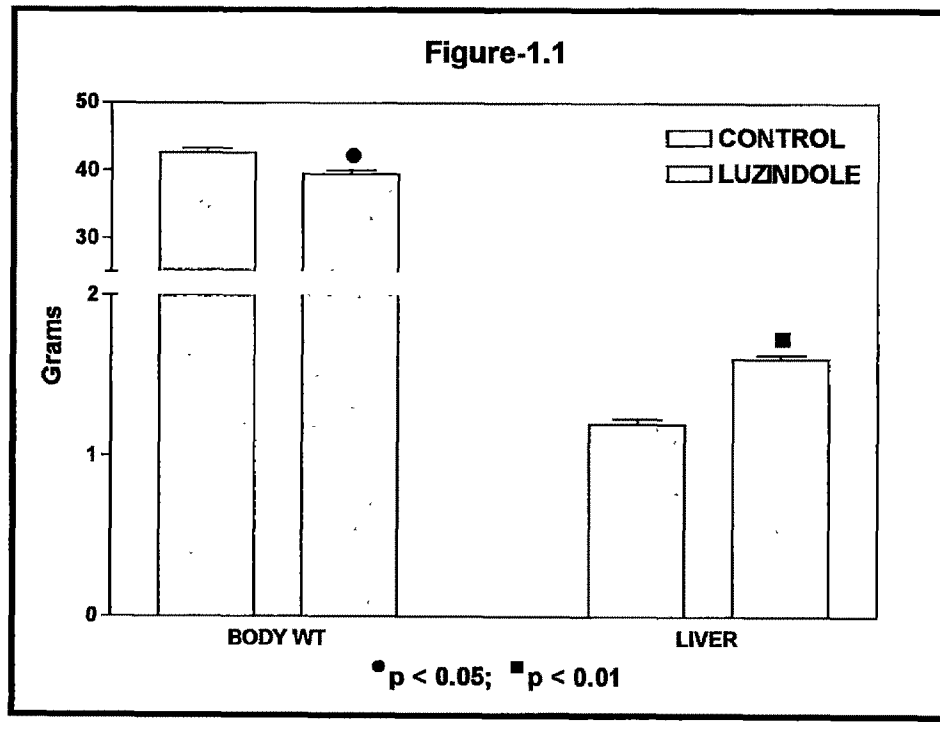


Table 1.1: Body and absolute weight of liver of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	CONTROL	LUZINDOLE
BODY WEIGHT	42.66 ±0.66	39.5• ±0.49
LIVER WEIGHT	1.20 ±0.034	1.61■ ±0.024

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

Figure 1.2: Absolute weights of pancreas, spleen, kidney, testes and adrenals of weaning rats on 22nd day subjected to neonatal luzindole treatment:

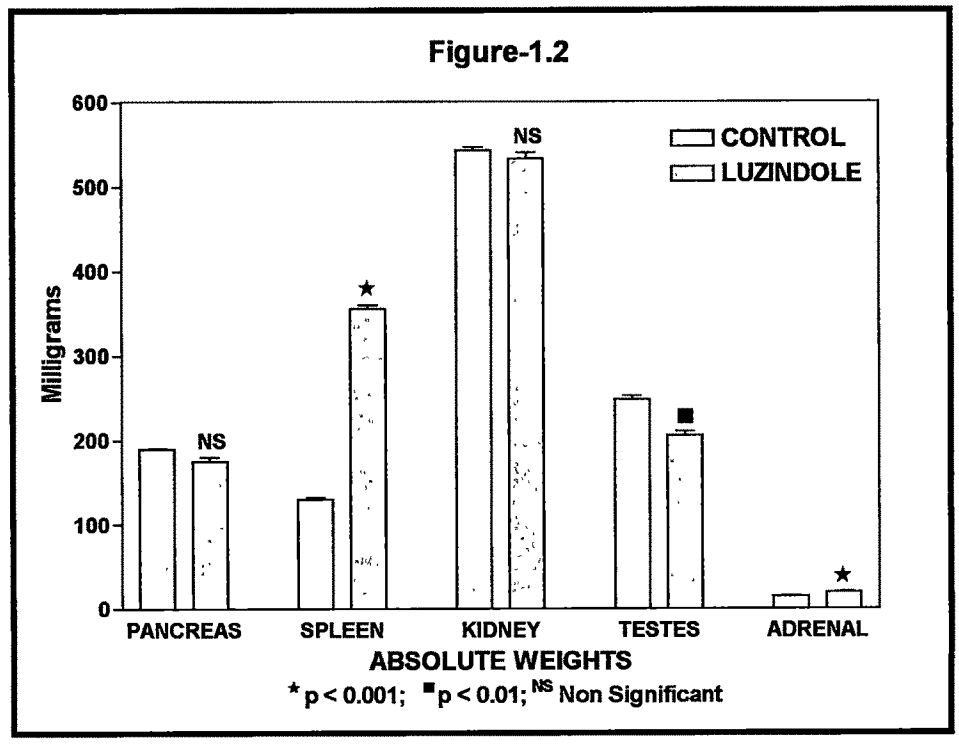


Table 1.2: Absolute weights of pancreas, spleen, kidney, testes and adrenals of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	PANCREAS	SPLEEN	KIDNEY	TESTES	ADRENAL
CONTROL	189.66 ±0.66	129.66 ±2.33	543.00 ±3.53	248.33 ±3.71	15.00 ±0.26
LUZINDOLE	175.00^{NS} ±5.01	355.00* ±5.01	532.5^{NS} ±7.51	205.00■ ±5.01	20.00* ±0.20

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

Figure 1.3: Relative weights of pancreas, spleen, testes and adrenals of weaning rats on 22nd day subjected to neonatal luzindole treatment:

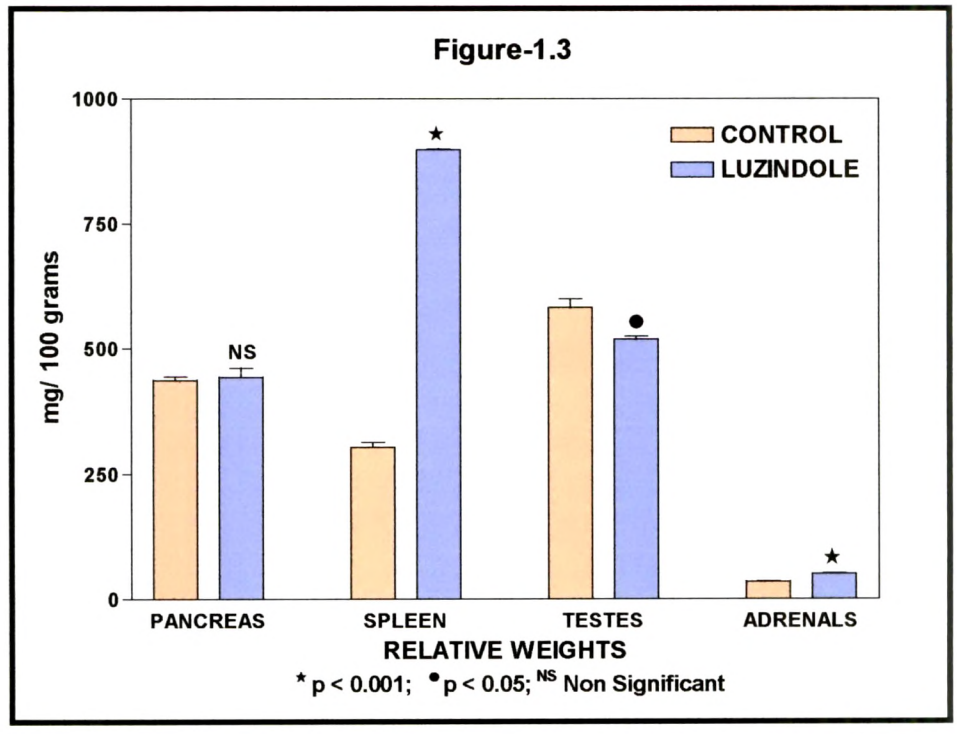


Table 1.3: Relative weights of pancreas, spleen, testes and adrenals of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	PANCREAS	SPLEEN	TESTES	ADRENAL
CONTROL	437.87 ±6.20	304.18 ±9.55	582.57 ±17.49	35.17 ±0.53
LUZINDOLE	443.86^{NS} ±18.31	898.71* ±1.28	518.91$^{\bullet}$ ±6.11	50.64* ±0.64

Values are expressed as mean ± SEM, $^{\star}p < 0.001$; $^{\bullet}p < 0.05$; ^{NS} Non Significant

Figure 1.4: Relative weight of liver and kidney of weaning rats on 22nd day subjected to neonatal luzindole treatment:

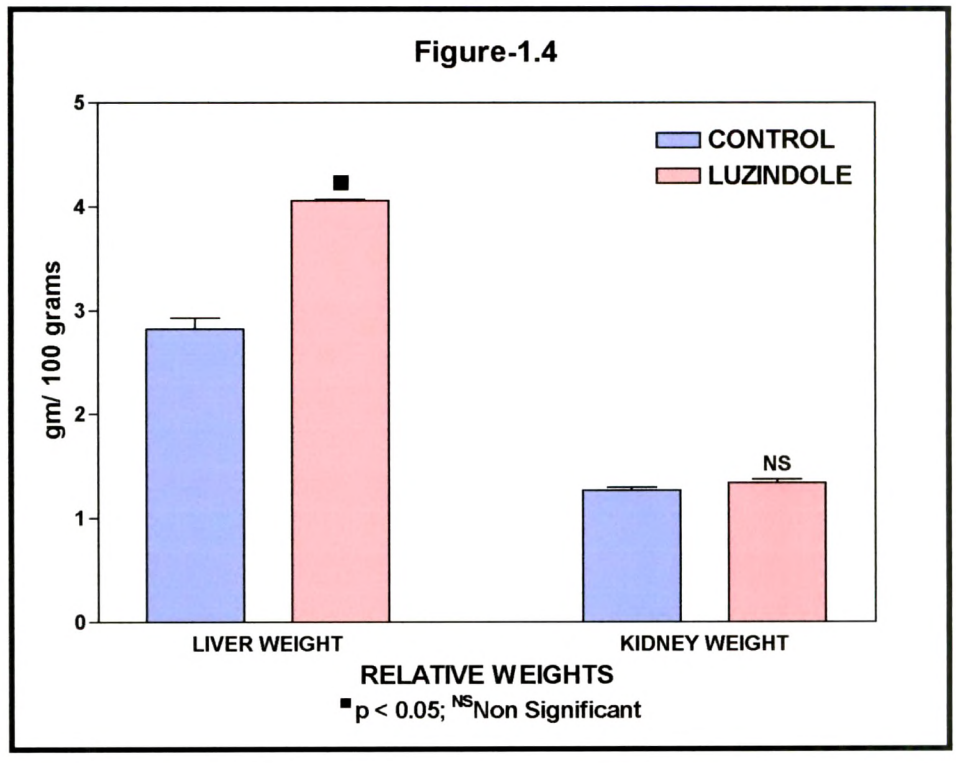


Table 1.4: Relative weight of liver and kidney of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	CONTROL	LUZINDOLE
LIVER WEIGHT	2.82 ±0.11	4.06• ±0.014
KIDNEY WEIGHT	1.27 ±0.024	1.34NS ±0.034

Values are expressed as mean ± SEM, •p < 0.05; NSNon Significant

Figure 1.5: Serum glucose and insulin levels of weaning rats on 22nd day subjected to neonatal luzindole treatment:

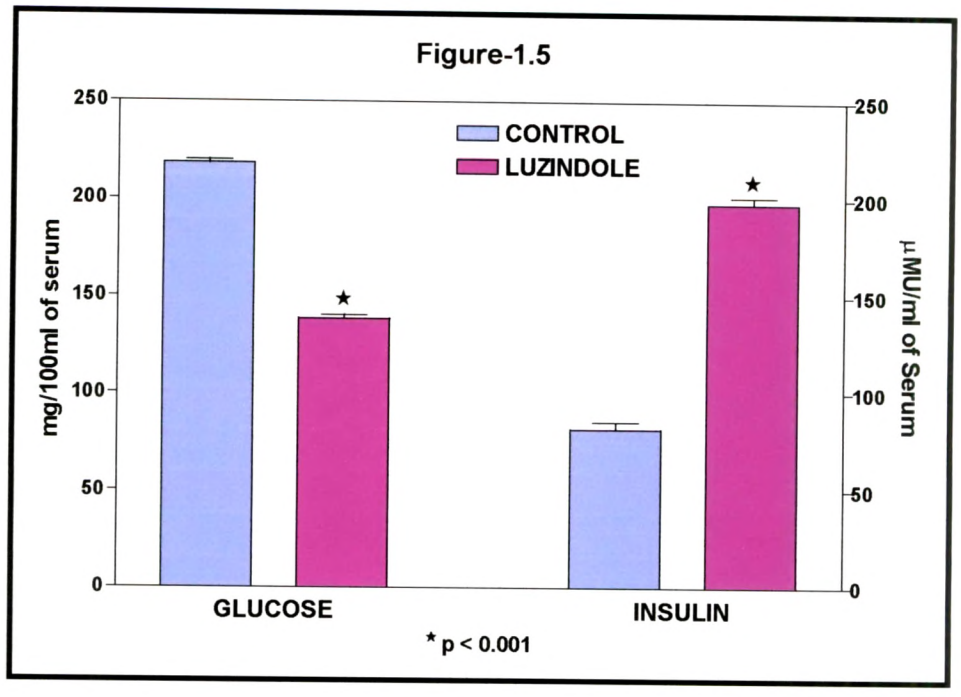


Table 1.5: Serum insulin and glucose levels of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	INSULIN	GLUCOSE
CONTROL	81.61 ±3.577	218.09 ±1.7567
LUZINDOLE	197.70* ±3.531	138.28* ±1.9213

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

Figure 1.6: Activity of glycogen phosphorylase in the liver and muscle of weaning rats on 22nd day subjected to neonatal luzindole treatment:

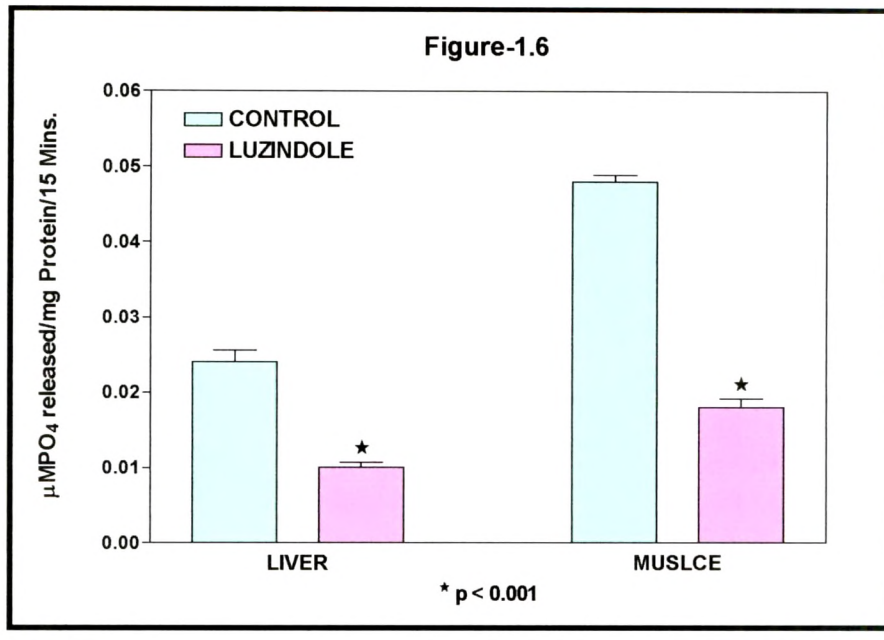


Table 1.6: Activity of glycogen phosphorylase in the liver and muscle of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	0.024 ±0.155	0.048 ±0.00085
LUZINDOLE	0.010* ±0.0007	0.018* ±0.0011

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

Figure 1.7: Glucose-6-phosphatase activity in the liver of weaning rats on 22nd day subjected to neonatal luzindole treatment:

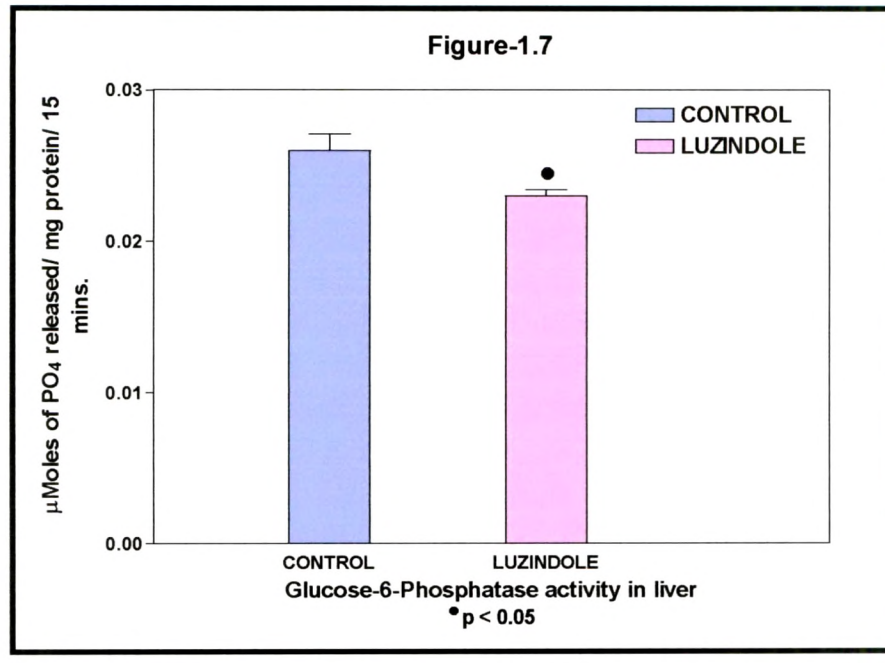


Table 1.7: Glucose-6-phosphatase activity in the liver of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	CONTROL	LUZINDOLE
GLUCOSE-6-PHOPHATASE	0.026 ±0.0011	0.023• ±0.00041

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

Figure 1.8: Glycogen synthetase activity in liver and muscle of weaning rats on 22nd day subjected to neonatal luzindole treatment:

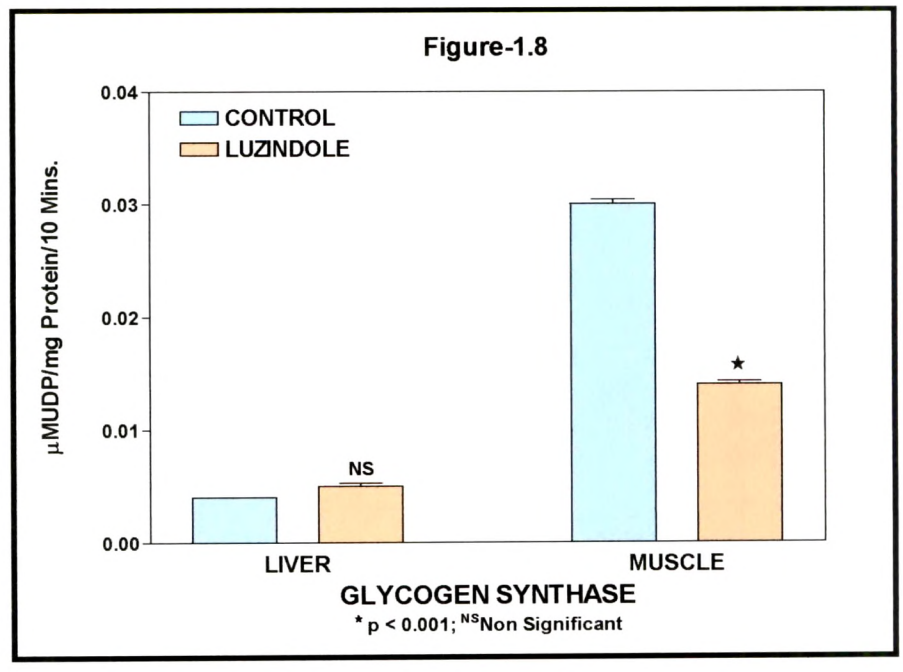


Table 1.8: Glycogen synthetase activity in liver and muscle of weaning rats on 22nd day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	0.004 ±0.000	0.030 ±0.000405
LUZINDOLE	0.005^{NS} ±0.0025	0.0140^{*} ±0.000405

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

Figure 1.9: Hepatic and muscle glycogen content of the weaning rats on 22nd day subjected to neonatal luzindole treatment:

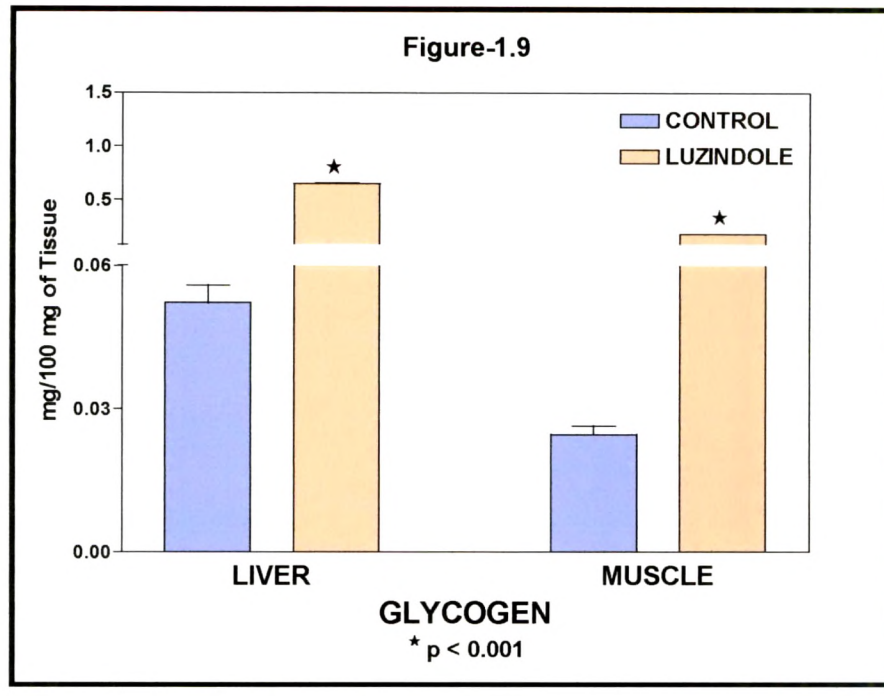


Table 1.9: Hepatic and muscle glycogen content of the weaning rats on 22nd day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	0.0522 ±0.0037	0.0247 ±0.00175
LUZINDOLE	0.649* ±0.0055	0.1778* ±0.001124

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

Figure 1.10: Hepatic and muscle protein content of the weaning rats on 22nd day subjected to neonatal luzindole treatment:

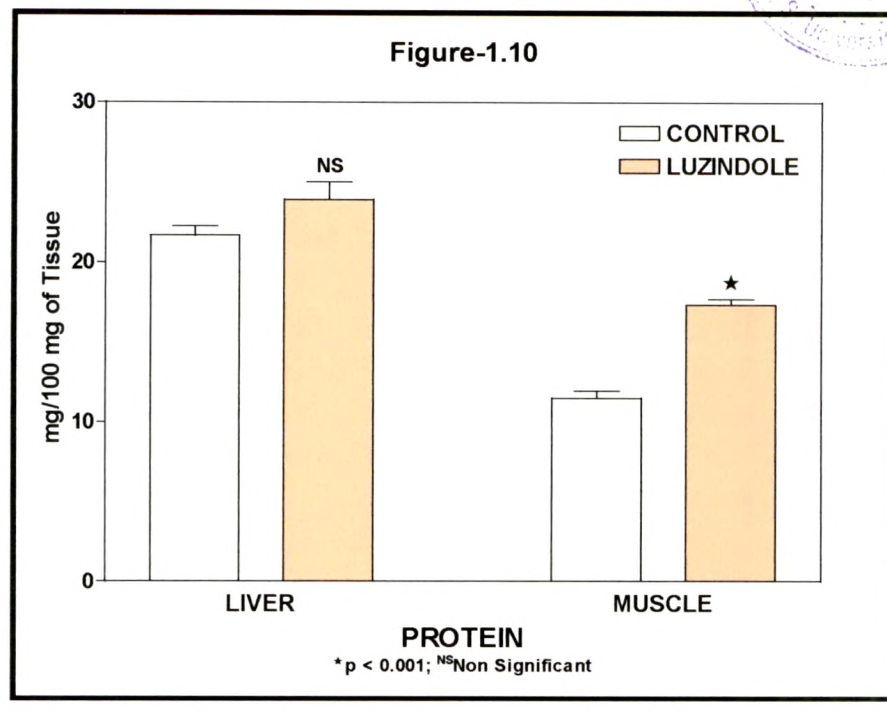


Table 1.10: Hepatic and muscle protein content of the weaning rats on 22nd day subjected to neonatal luzindole treatment:

	LIVER	MUSCLE
CONTROL	21.66 ±0.5915	11.49 ±0.44
LUZINDOLE	23.91 ±1.075	17.33 ±0.3603

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

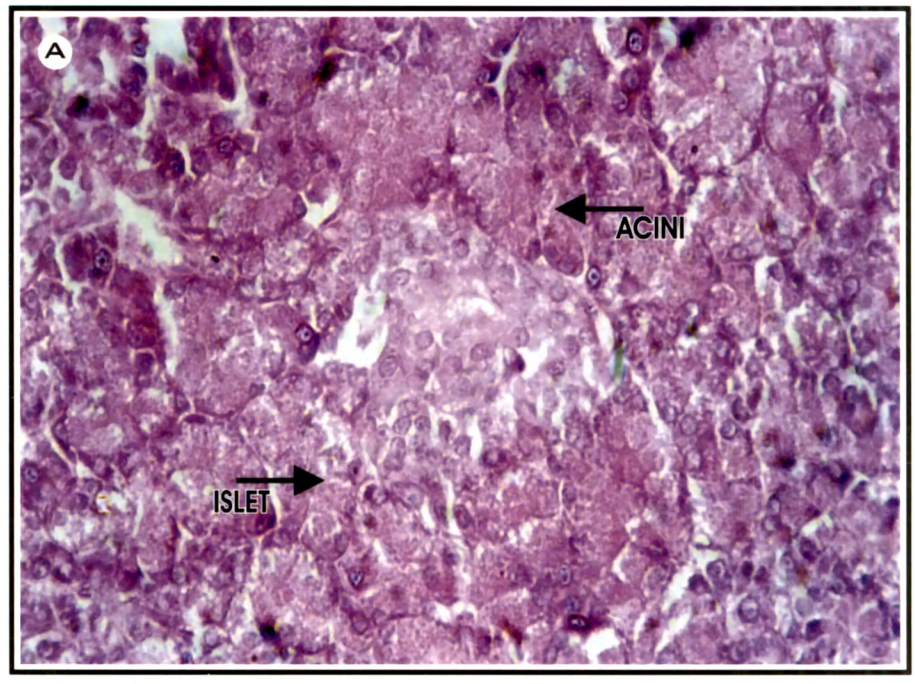
PLATE – 1

Photomicrographs of sections of pancreas – 450 X

FIGURE (A): Transverse section of the pancreas of male control weaning (22^{nd} day) rats showing islet and pancreatic acini. Note the centrally distributed A cells, peripherally distributed B cells.

FIGURE (B): Transverse section of the pancreas of male luzindole treated rats on the 22^{nd} 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 - 1



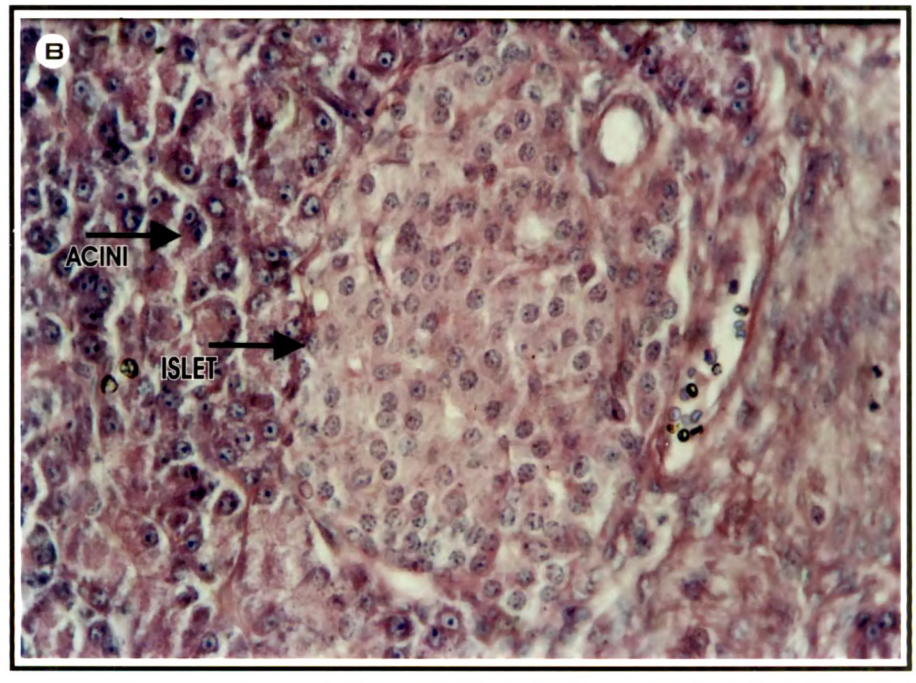


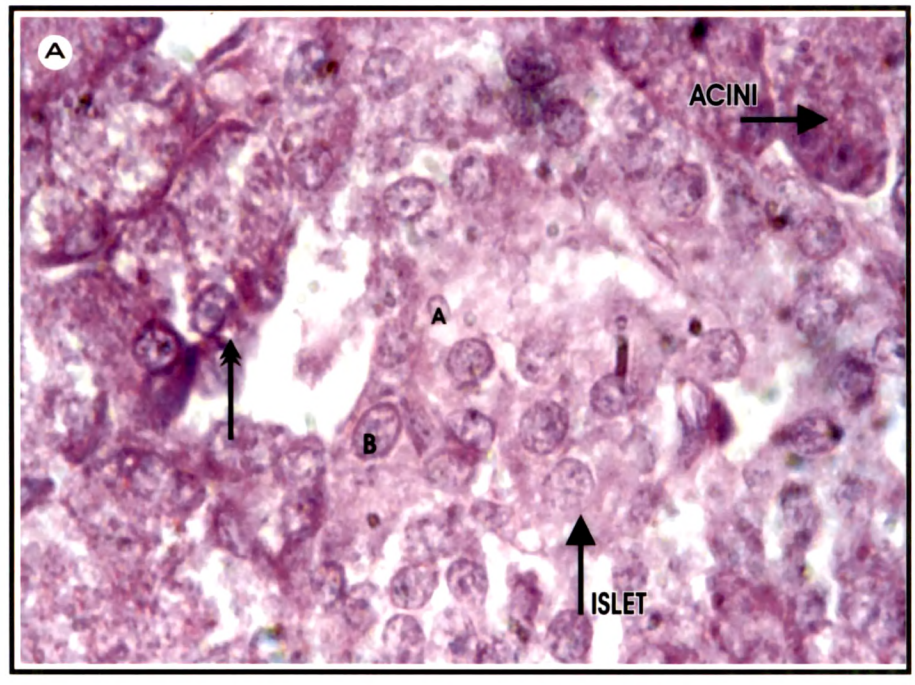
PLATE – 2

Photomicrographs of sections of pancreas – 1000 X

FIGURE (A): Transverse section of the pancreas of male control weaning (22^{nd} day) rats showing islet and pancreatic acini. Note the centrally distributed A cells, peripherally distributed B cells and the transdifferentiating cell (double headed arrow).

FIGURE (B): Transverse section of the pancreas of male luzindole treated rats on the 22^{nd} day showing islet and pancreatic acini. The number of B cells in the islet is increased as compared to the A cells. Note the transdifferentiating cells (double headed arrow) on the periphery of the islets

PLATE - 2



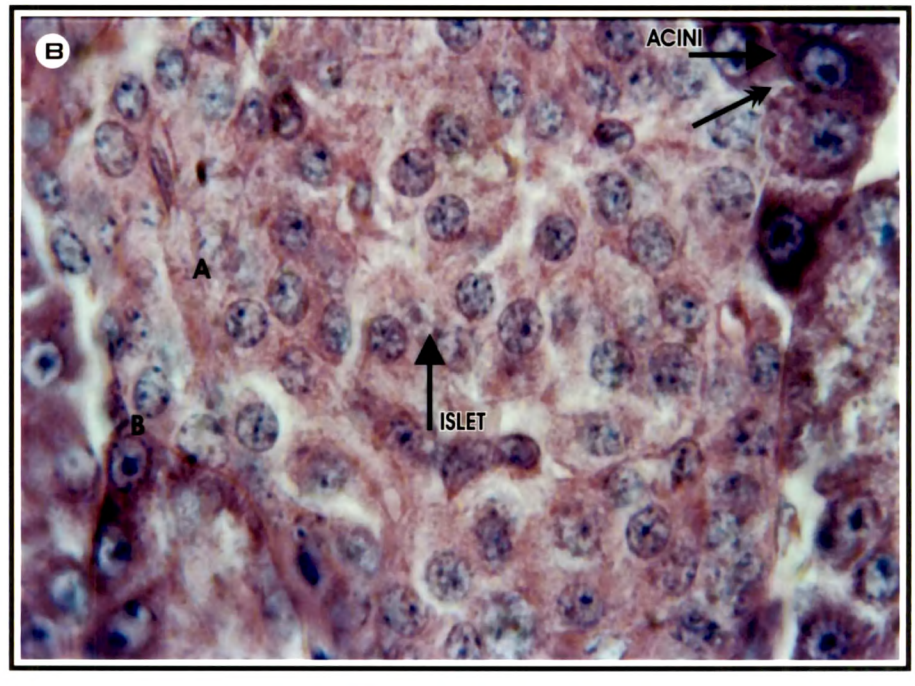


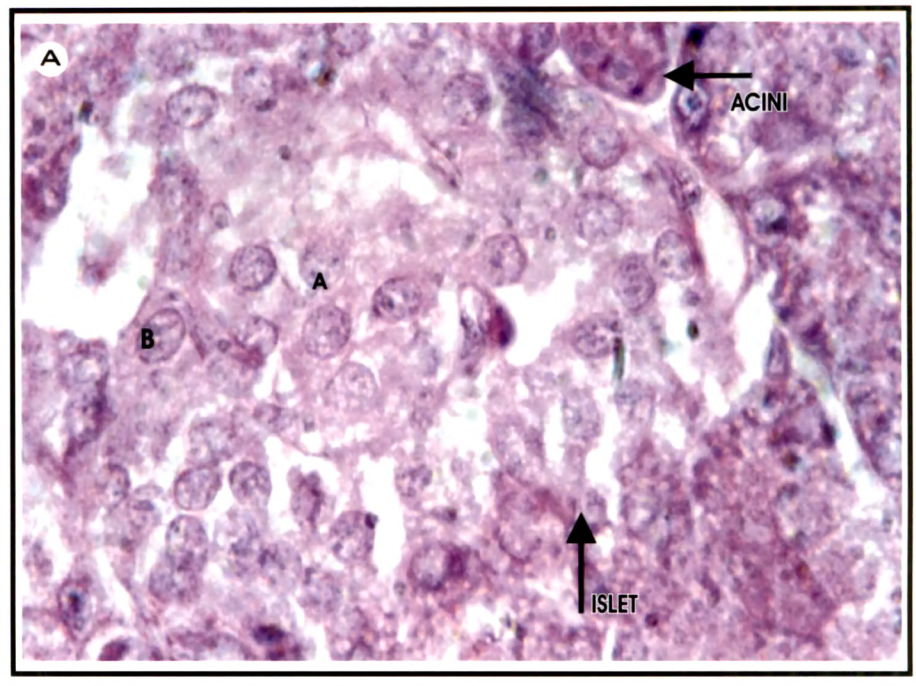
PLATE – 3

Photomicrographs of sections of pancreas – 1000 X

FIGURE (A): Transverse section of the pancreas of male control weaning (22^{nd} 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 22^{nd} day showing islet and pancreatic acini. The number of B cells in the islet is increased as compared to the A cells. Note the transdifferentiating cells (double headed arrow) on the periphery of the islets such that the area between the acini and the islet is indistinguishable.

PLATE - 3



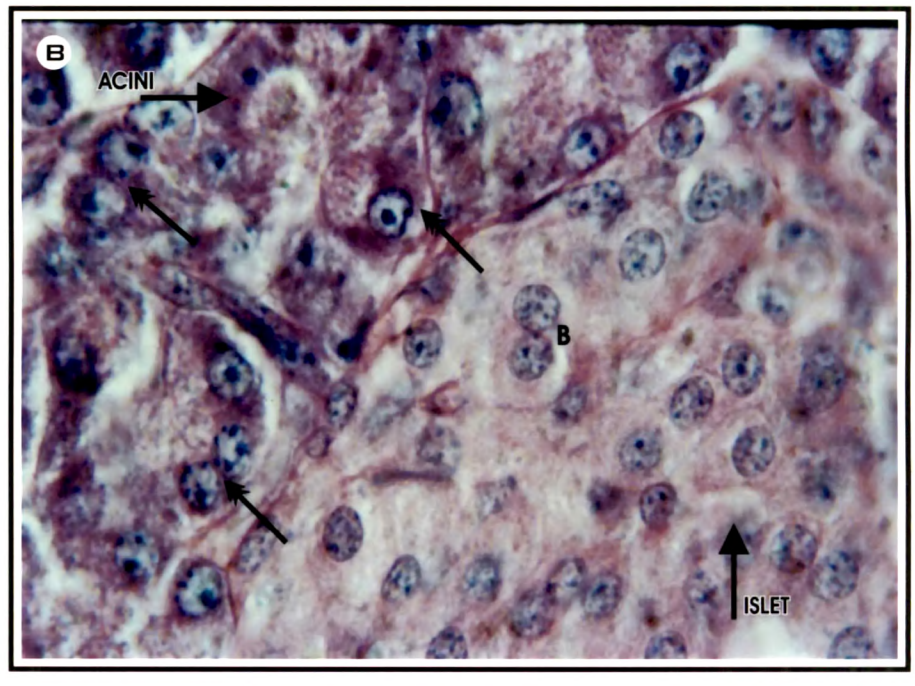


PLATE – 4

Photomicrographs of sections of pancreas – 1000 X

FIGURE (A), (B) & (C):

Transverse section of the pancreas of male luzindole treated (22^{nd} day) rats showing islet and pancreatic acini. Note the transdifferentiating (double headed arrow) cells on the periphery of the islet. There is an significant increase in the B cell number as compared to the A cells.

PLATE - 4

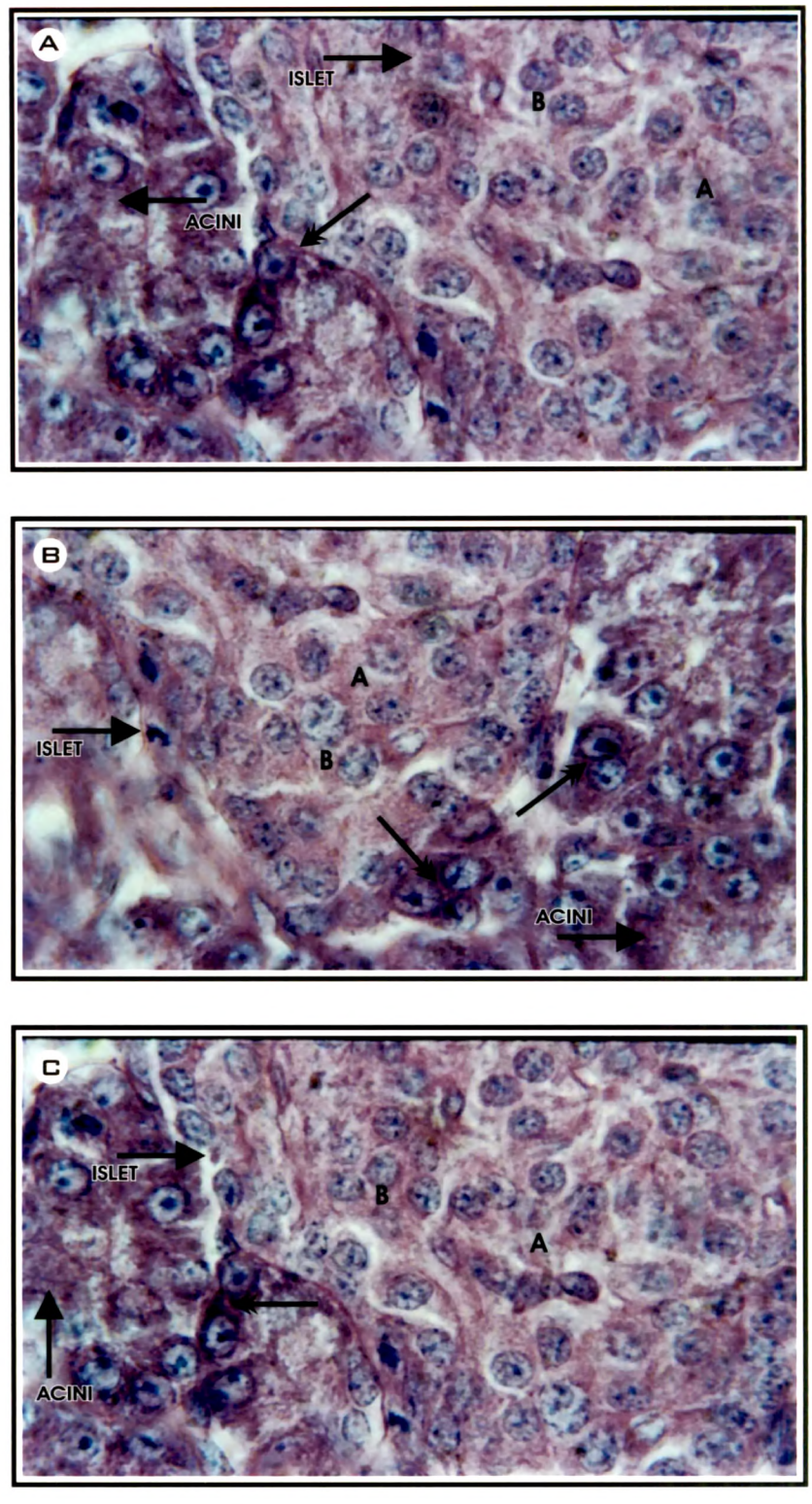


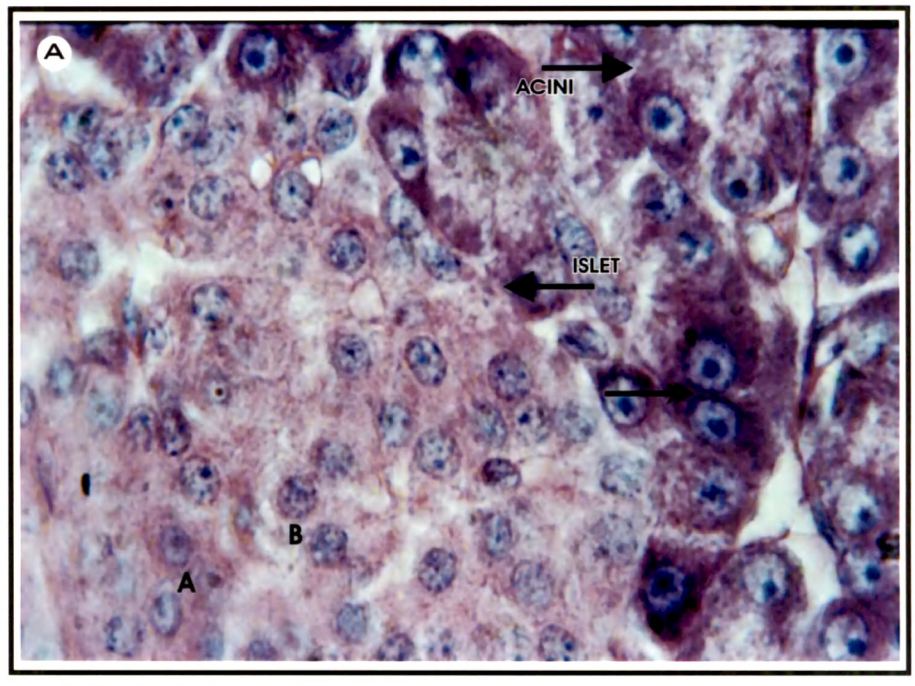
PLATE – 5

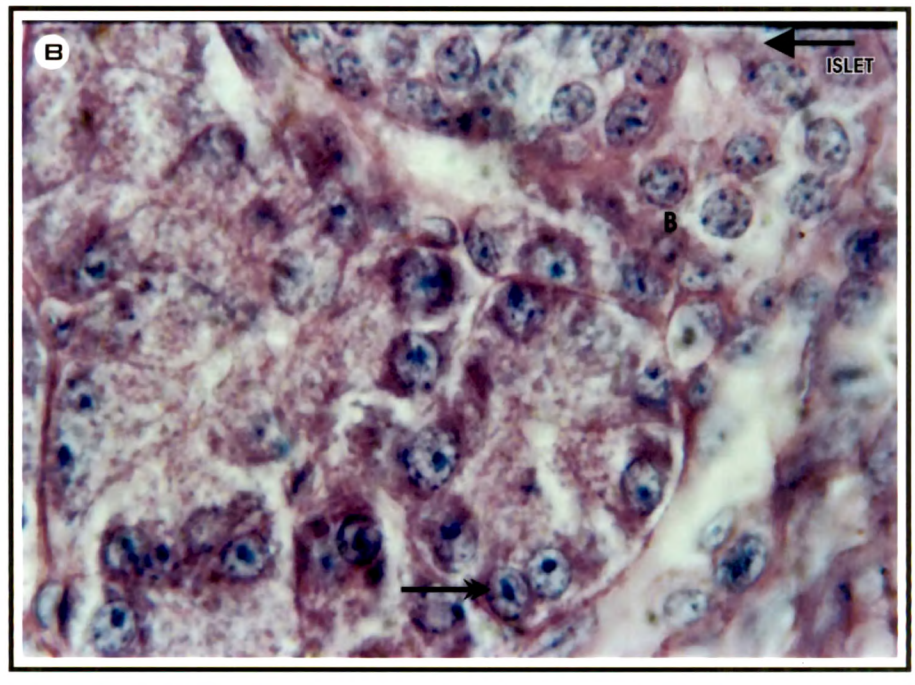
Photomicrographs of sections of pancreas – 1000 X

FIGURE (A) & (B):

Transverse section of the pancreas of male luzindole treated rats on the 22nd day showing islet and pancreatic acini. The number of B cells in the islet is increased as compared to the A cells. Note the transdifferentiating cells (double headed arrow) on the periphery of the islets such that the area between the acini and the islet is indistinguishable.

PLATE - 5





- **Hepatic Glycogen content and activities of Glycogen Synthetase, Glycogen Phosphorylase and Glucose-6-Phosphatase:** The hepatic glycogen content showed a significant increase while there was no alteration in the glycogen synthetase activity. The glycogen phosphorylase and glucose-6-phosphatase decreased significantly in the luzindole treated animals (Fig. and Tab. 1.6, 1.7, 1.8 and 1.9).
- **Muscle Glycogen content and activities of Glycogen Synthetase and Glycogen Phosphorylase:** The muscle glycogen content showed a significant increase while the glycogen synthetase and glycogen phosphorylase activity were significantly decreased in the luzindole treated animals (Fig. and Tab. 1.6, 1.8 and 1.9).
- **Hepatic and muscle protein content:** The muscle protein content was significantly increased whereas the hepatic protein content showed only a marginal increase although non significant in the luzindole treated animals as compared to controls (Fig. and Tab. 1.10).
- **Histological observations:** Histologically, the pancreatic islets of L22 animals show higher number of B cells and lesser number of A cells compared to control islets. There appears to be some degree of transdifferentiation of acinar cells to islet cells. The ratio of B:A cells is also significantly higher in the luzindole treated rats (Plate; 1-5).

DISCUSSION:

Though the modulatory influence of melatonin an carbohydrate metabolism has been studied to a greater extent in adult animals (Delahaunty *et al.*, 1978; Dhar *et al.*, 1983; Mahata *et al.*, 1988; John *et al.*, 1990; Zemen *et al.*, 1993; Ramachandran, 2002), the involvement of this hormone in neonatal physiology is never studied. A current study on this aspect from this laboratory has clearly brought out the impact of neonatal hypermelatonemia on insulinemia, glycemia and tissue carbohydrate metabolism (Jani, 2004). The present study is the first one which has attempted to see the effect of induced functional hypomelatonemia in the neonatal period by using the melatonin receptor (MT_2) antagonist, luzindole on carbohydrate homeostasis. The results obtained clearly show increased tissue glycogen content with decreased synthetase to phosphorylase activity ratio and hypoglycemia coupled with hyperinsulinemia. The hepatic and muscle glycogen content have shown a tremendous increase of more than one thousand and six hundred percentage respectively. Decreased glycogen phosphorylase activity coupled with increased synthetase activity resulting in a higher synthetase: phosphorylase activity ratio seem to be the causative factor for the observed glycogenic effect. However the degree of tissue glycogen accumulation obtained with luzindole appears to be significantly higher than that seen with hypermelatonemic state (Jani, 2004). Whereas the glycogenic effect seen in the above study was explained as due to significantly increased insulin sensitivity despite a hypoinsulinemic state, the

present effect can be considered to be due to the recorded significant hyperinsulinemia. The unaltered glucose-6-phosphatase activity in luzindole treated rats compared to controls is also supportive of the hyperinsulinemic glycogenic state. The observed decrease in phosphorylase activity and increase in synthetase activity are indicative of the hyperinsulinemic effect. Nevertheless, the synthetase: phosphorylase activity ratio is much lower than that seen with hypermelatonemic state and hence presents a paradox. Though no possible explanation could be provided at this moment, a likelihood of luzindole directly promoting glycogenesis by as yet unknown mechanisms may have to be investigated. This suggestion is supported by a recent study of Zhou *et al.* (2003) on the effect of luzindole on voltage activated transient outward K^+ current in rat cerebellar granule cells wherein, they have opined that except blocking receptor, some receptor antagonist might have other biological effects, such as blocking channels, activating or inhibiting enzymes and so on. Similar suggestion has also been made by Lu and Hu. (2002) on hyperzine A, a nootopic agent. It is this possible effect of luzindole and the hyperinsulinemic state that may have to be taken together to account for the presently recorded glycogenic influence as against the report of pinealectomy induced decrease in hepatic and muscle glycogenesis (Milcu *et al.*, 1971; Mellado *et al.*, 1986). Another valid point to be considered is the age of the animals used; whereas the above studies have used adult rats, the present study involves neonates. Obviously the age specific differences in response as well

as sensitivity could also be a factor of significance in interpreting the results. The hypoglycemic status together with hyperinsulinemia also provides a compelling evidence for glucose transport and glycogenesis. Since a study on *in vitro* glucose uptake by liver and muscle slices of luzindole treated rats has shown decreased sensitivity to stimulatory agents like insulin, acetylcholine and melatonin (Chapter-3) the mechanism of glucose transport and glycogenesis is not clear. A possible direct but unknown action of luzindole other than its role as a melatonin receptor antagonist may have to be evaluated. Though there are studies on melatonin administration and pinealectomy, which show altered insulinemia and glycemia and insulin resistance (Fabis *et al.*, 2002; Picinato *et al.*, 2002; Zanquetta *et al.*, 2003), there are no studies on functional hypomelatonemia which preclude making meaningful discussion. In this connection, the present study has shown novel changes in carbohydrate homeostasis with the use of a melatonin receptor blocker. Probably more studies are needed to understand the intricacies of effect of melatonin blockage and even probably the direct action of luzindole other than its receptor antagonism.

Concurrent to the changes in carbohydrate metabolism, luzindole treatment has also differential effects on hepatic and muscle protein contents. Whereas there was no change in hepatic protein content, the significant increase in muscle protein content could be related with the prevailing hyperinsulinemia induced anabolic status. The relative organ weights in luzindole treated rats have shown significant increase

with reference to pancreas, liver, spleen, kidney and adrenals. Presumably, organ growth seems to be promotive under conditions of blockage of melatonin action. The higher serum insulin level is well corroborated by the observed higher number of B cells and higher B:A cell ratio in the pancreatic islets of luzindole treated rats. Increased transdifferentiation of acinar cells also seems to be a feature of neonatal melatonin blockage (Plate; 1-6). Organ growth kinetics could be an area of investigation under altered melatonin status in the neonatal period. It can be concluded from present study that blockage of melatonin action during the neonatal period has significant influence on systemic carbohydrate homeostasis marked by hyperinsulinemia, hypoglycemia and increased glycogenesis. Possible involvement of luzindole in functions related to carbohydrate metabolism by as yet unknown mechanisms, other than its role as a receptor antagonist is also suspected, and more studies are needed in this direction to clarify the action.

SUMMARY:

The effect of an altered melatonin status in the neonatal period had shown decreased serum insulin level, increased insulin sensitivity and altered carbohydrate homeostasis. The present study in this backdrop has been designed to test the effect of blockage of melatonin action on serum insulin and glucose levels and, hepatic and muscle carbohydrate homeostasis. 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 22^{nd} day. There is a significant decrease in the body weight of luzindole treated (L22) rats while, the relative weights of pancreas and kidneys showed no significant alterations as compared to the controls. The relative weights of liver, spleen and adrenals increased significantly and that, of testes decreased significantly in the L22 rats. The serum glucose level decreased while, the serum insulin level increased significantly in the experimental rats. The hepatic and muscle glycogen contents increased significantly in the L22 rats whereas, the activities of glycogen synthetase and glycogen phosphorylase decreased significantly in the muscle of the experimental rats through the, activity of glycogen synthetase in liver remained unaltered as compared to the controls. The activities of glycogen phosphorylase and glucose-6-phosphatase decreased significantly in the liver of L22 rats. The muscle protein content of the experimental rats decreased significantly while, the hepatic protein content showed no significant alteration as compared to the control

rats. Histologically, the pancreatic islets of L22 rats showed higher number of B cells and lesser number of A cells compared to control islets. There appears to be some degree of transdifferentiation of acinar cells to islets cells in the pancreatic islets of L22 rats. It can be concluded from the present study that, blockage of melatonin action during the neonatal period has significant influence on systemic carbohydrate homeostasis marked by hyperinsulinemia, hypoglycemia and increased glycogenesis. Possible involvement of luzindole in functions related to carbohydrate metabolism by as yet unknown mechanisms, other than its role as a receptor antagonist, is also suspected, and more studies are needed in this direction to clarify the action.