DIVISION I

TYPE I DIABETES IN RELATION TO FEMALE SEX STEROIDS: MELATONIN AS AN ALTERNATIVE THERAPY FOR TYPE I DIABETES IN SEX STEROID DEFICIENCY

MELATONIN SUPPLEMENTATION THERAPY (MST) AS A POTENT ALTERNATIVE TO ERT IN ALLEVIATING ALTERED INSULIN SENSITIVITY, GLUCOREGULATION AND DYSLIPIDEMIA: DOSE DEPENDENT STUDY IN ESTROGEN DEFICIENT OVARIECTOMIZED RATS

There is an apparent rise in the observed incidence of diabetes, osteoporosis and cardiovascular diseases in women in the age group of 35-55 years as compared to men and, this is mainly attributed to the decreasing levels of sex steroids as a prelude to the onset of menopause. Menopause, may be considered to be a mid life event that brings along with it a series of physiological changes that makes the individual susceptible to various diseases due to the changing internal *milieu*. There is reduced pancreatic secretion during menopause but little change is observed in the overall circulating insulin level as a result of reduced elimination of the secreted insulin (Perera and Wedisinghe, 2009). However, with increase in age, there is a progressive increase in insulin resistance that predisposes women in post menopausal period to the development of Type II diabetes. There is a reduction in whole body insulin mediated glucose uptake associated with the loss of ovarian function (Proudler *et al.*, 1992) and these changes that accompany menopause make the condition even worse in women with Type I diabetes and may further hamper glycaemic regulation and reduce insulin sensitivity (Stromeyer *et al.*, 2003).

Hormone replacement therapy (HRT), advised by clinicians to overcome the signs and symptoms of menopause, comes with a cost and results obtained from long term studies have not been convincing

enough to propagate the use of HRT for every individual *per se* (Perera and Wedisinghe, 2009). There is a reported eight fold increase in incidence of endometrial cancer in women who are on a prolonged unopposed Estrogen therapy (Grady *et al.*, 1995; McPherson *et al.*, 1996). The benefit of Estrogen being used in HRT is somewhat disputed with reports indicating lower doses to have a beneficial effect on insulin sensitivity, while higher doses having a detrimental effect on insulin sensitivity (Ajabar *et al.*, 1972; Cagnacci *et al.*, 1992). There are reports that Medroxyprogesterone acetate results in deterioration of insulin resistance (Goldsland *et al.*, 1993; Lindheim *et al.*, 1993) while, dyhydroprogesterone in combination with estradiol is potent enough to reverse menopause associated changes in insulin secretion and elimination (Godsland *et al.*, 2004; Dansuk *et al.*, 2005). These reports are thus suggestive of requirement for a stringent regulation of HRT in terms of dosage, time and duration of administration. From the point of view of regulation of lipid and carbohydrate metabolism in menopausal women, it is difficult to draw a finish line as of now with the available studies on recommendation of appropriate doses and use of HRT in such patients. To this end, there is a need to develop non estrogen based treatments for menopausal symptoms.

Melatonin shows a circadian rhythm, with a peak at night. An interesting observation seen in night shift workers is clearly indicative of the important role of melatonin in maintaining metabolic functions in the body. Night shift workers, have shown higher levels of insulin, glucose and triacylglycerols after a night time meal than after a daytime meal, which suggests the desynchronization of bodily functions combined with a higher

incidence of heart disease and metabolic disturbances like diabetes (Morgan *et al.*, 2003). Role of melatonin in stimulating glucose transport mediated through IRS I / PI-3-kinase pathway in skeletal muscles is implicative of its potential role in maintaining glucose homeostasis and possibly in diabetes (Patel and Ramachandran, 1992; Ha *et al.*, 2006; Singh *et al.*, 2010 b). Exposure to light at night and aging, both of which lower melatonin levels have been suspected to be contributing to the incidence and / or development of diabetes.

Therefore, the present study is one such attempt to understand the role of an alternative therapeutic (Melatonin) using two different doses in sex steroid deficiency and a comparison thereat with its efficacy in relation to ERT given alone or in combination on various parameters of carbohydrate and lipid metabolism in ovariectomized *Wistar* rats, taken as model for female hormone deficiency or menopause.

RESULTS

Body weight gain, feed intake and efficiency and water intake (Table 1A, 1B, Fig 1)

Table 1A and B shows the changes in food and water intake, body weight gain and feed efficiency at the end of the 5 weeks treatment in sham operated and OVX animals. Compared to the sham operated control animals, OVX animals showed an increase in body weight gain alongwith increased feed efficiency. Administration of estrogen and/or melatonin significantly reduced the ovariectomy induced increase in body weight gain and food intake. Out of the two doses of melatonin used in the present study, MH singly

and in combination with E_2 showed the greatest decrement in feed efficiency and body weight gain as compared to all the other treatment schedules. Progesterone supplementation showed a similar trend of decrement in body weight gain and feed efficiency.

Relative organ weights (Table 2 and Fig 2)

Table and Fig 2 depict the relative organ weights of liver, muscle, kidney, uterus and adipose of all the experimental groups. There was a marked increment in the relative weight of adipose tissue in OVX animals compared to the sham operated control animals. This increase was significantly reverted back by melatonin administered alone or in combination with estrogen and was much more significant compared to the decrease brought about by estrogen/ progesterone treatment individually. OVX animals showed a significant decrement in the uterine weight and estrogen treatment either singly or in combination with both the doses of melatonin showed significant increment in the uterine weights. Relative weights of liver, muscle and kidney were also increased in OVX animals but were statistically insignificant.

Serum glucose, insulin and fasting insulin resistance index (FIRI) (Table 3A and Fig 3.1, 3.2, 3.3)

Table 3A depicts the fasting serum glucose and insulin levels alongwith the FIRI of all the groups. Ovariectomy did not have any significant effect on the glycaemic status but showed a significant increase in insulin titre compared to the sham operated control group. There was significant insulin resistance as marked by increased FIRI value in these animals. Both estrogen and progesterone treatments though significantly reduced the serum insulin titres

and FIRI values in OVX animals, were not however successful in reverting to the levels of control animals. On the other hand, both doses of melatonin brought down the insulin levels significantly with the higher dose being more effective. There was a corresponding decrease in the FIRI values of these groups in consequence. The combinational treatment of estrogen and melatonin showed changes similar to those of melatonin alone with E_2+ML being maximally effective.

Serum hormone profile of Estrogen and Progesterone (Table 3B and Fig 3.4, 3.4)

Table 3B also shows the circulating sex steroid levels in all the experimental groups. OVX animals showed significant decrease in estrogen and progesterone levels with only traces of these hormones remaining in the circulation as a result of ovariectomy in comparison to the ovary intact sham operated control animals. There was significant increment in the estrogen level in E_2 replaced groups (OVX+E_2, OVX+E_2+ML, OVX+E_2+MH) owing to exogenous supplementation with estrogen. Correspondingly, progesterone levels increased significantly in the OVX+P_4 group due to the exogenous progesterone supplementation.

Oral glucose tolerance and Insulin response tests and, Insulin sensitivity index (Table 4.1, 4.2, 5.1, 5.2 and Fig 4.1, 4.2, 5.1, 5.2)

Figures 4.1 and 5.1 depict the GTT and IRT curves in all the experimental groups while; Fig 4.2 represents area under the curve for GTT and Fig 5.2 the insulin sensitivity index. Ovariectomy showed an increased area under curve in comparison to the sham operated control. Melatonin treatment (both doses)

could significantly reduce the area under curve with a bettered glucose tolerance. However, combinations of E_2+M showed maximal effect in terms of reduced area under the curve. Progesterone however did not show any betterment in GTT.

Figure 5.1 depicts insulin response curves while fig 5.2 shows the insulin sensitivity index of all groups of animals. Except for the IR curves of OVX, OVX+P_4 and OVX+MH which were poorer compared to control animals, the other curves of all the other experimental groups showed bettered IR curves with OVX+E_2+ML and OVX+ML being the best in that order followed by OVX+E_2+MH and OVX+E_2. The insulin sensitivity index as represented by the K_{IS} values (Fig 5.2) also reflected the same with maximal sensitivity being rended for OVX+E_2+ML followed by OVX+ML, OVX+E_2+MH and OVX+E_2 respectively. The poorest K_{IS} values were obtained for OVX, OVX+P_4 and OVX+MH.

Carbohydrate metabolism (Table 6, 7 and Fig 6.1, 6.2, 6.3,7.1, 7.2, 7.3)

Table 6 shows hepatic glycogen content and activity levels of glycogen phosphorylase and glucose-6-phosphatse. Ovariectomized animals showed significant decrease in hepatic glycogen content and significant increment in the activity levels of both the enzymes. In general, except for OVX, OVX+P_4 and OVX+MH groups of animals which showed similar changes of decreased glycogen content and increased phosphorylase and G-6-Pase activities, all other groups of animals effectively reversed the OVX induced changes with effectiveness being in the order E_2 > E_2+ML>ML> E_2+MH.

Table 7 shows the muscle glycogen content alongwith phosphorylase activity. Ovariectomy significantly decreased the muscle glycogen content and increased glycogen phosphorylase activity as compared to the sham operated control. Similar to the hepatic changes, even muscle glycogen content and phosphorylase activity were the poorest in OVX, OVX+P_4 and OVX+MH groups of animals. The most favourable changes in terms of reversal of OVX induced changes were seen in ML followed by E_2, E_2+ML and E_2+MH.

Serum lipid profile: (Table 8 and Fig 8)

Table 8 depicts the serum lipid profile in all the experimental groups. There is a marked increase in the serum levels of TC, TG, LDL and VLDL with a corresponding decrease in HDL in ovariectomized animals compared to the sham operated control animals. Both estrogen and progesterone supplementation reverted the alterations observed in the OVX animals though still not totally to the control levels. Melatonin treatment both individually and in combination with E_2 could positively regulate the changes in OVX animals with MH being maximally effective followed by E_2+MH, ML and E_2+ML in that order.

Tissue cholesterol and lipid content (Liver, Muscle and Kidney) (Table 9 A and B, Fig 9.1, 9.2)

Table 9 shows the tissue cholesterol and lipid contents in all the experimental groups. OVX animals showed increment in cholesterol and lipid contents in all the three tissues. Estrogen though could significantly regulate the increase in tissue cholesterol content, was nevertheless less effective in reversing tissue lipid contents. Treatment with high dose of melatonin (OVX+MH and

OVX+E_2+MH) could normalize the tissue cholesterol and lipid contents most significantly. Melatonin at low dose was also efficient but less significantly as compared to the higher dose.

Table 1A and B: Body weight, Food intake, water intake and feed efficiency in all the experimental groups

(A)

GROUPS	Food Intake (g/animal/day)	Water Intake (ml/animal/day)
SO	17.55±1.23	36.12±6.32
OVX	28.23±2.45^c	40.32±3.45
OVX +E_2	18.25±3.12*	35.56±2.56
OVX +P_4	24.54±2.58^c	38.88±4.12
OVX +ML	19.23±2.65*	32.21±3.87*
OVX +MH	17.45±2.45*	30.54±3.44*
OVX +E_2+ML	15.65±1.11^e	35.01±2.56
OVX +E_2+MH	13.33±1.02$^@$	36.00±2.12

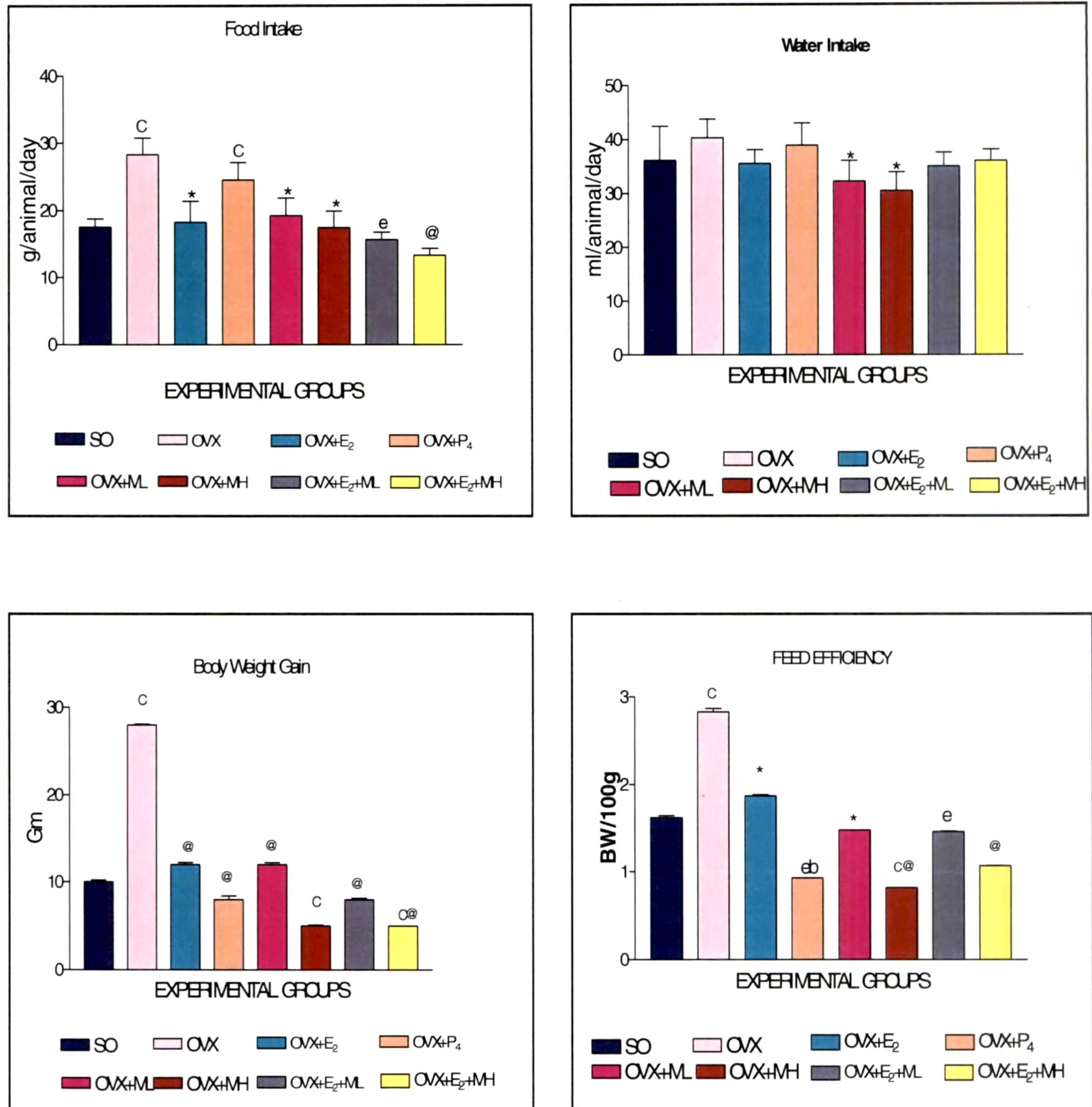
Data are expressed as Mean±SE
^{c}p<0.001 when compared to sham operated control and *p<0.05, ^{e}p<0.01, $^@$p<0.001 when compared to ovariectomized animals.

(B)

GROUPS	Body weight gain g	Feed efficiency BW/100g food intake
SO	10.00±0.21	1.62±0.022
OVX	28.00±0.12^c	2.83±0.04^c
OVX +E_2	12.00±0.35$^@$	1.87±0.014*
OVX +P_4	8.00±0.44$^@$	0.93±0.001^{eb}
OVX +ML	12.00±0.24$^@$	1.48±0.002*
OVX +MH	5.00±0.102^c	0.82±0.003$^{c@}$
OVX +E_2+ML	8.00±0.17$^@$	1.46±0.005^e
OVX +E_2+MH	5.00±0.02$^{c@}$	1.07±0.004$^@$

Data are expressed as Mean±SE
^{b}p<0.01, ^{c}p<0.001 when compared to sham operated control and *p<0.05, ^{e}p<0.01, $^@$p<0.001 when compared to ovariectomized animals.

Figure 1: Body weight, Food intake and water intake in all the experimental groups



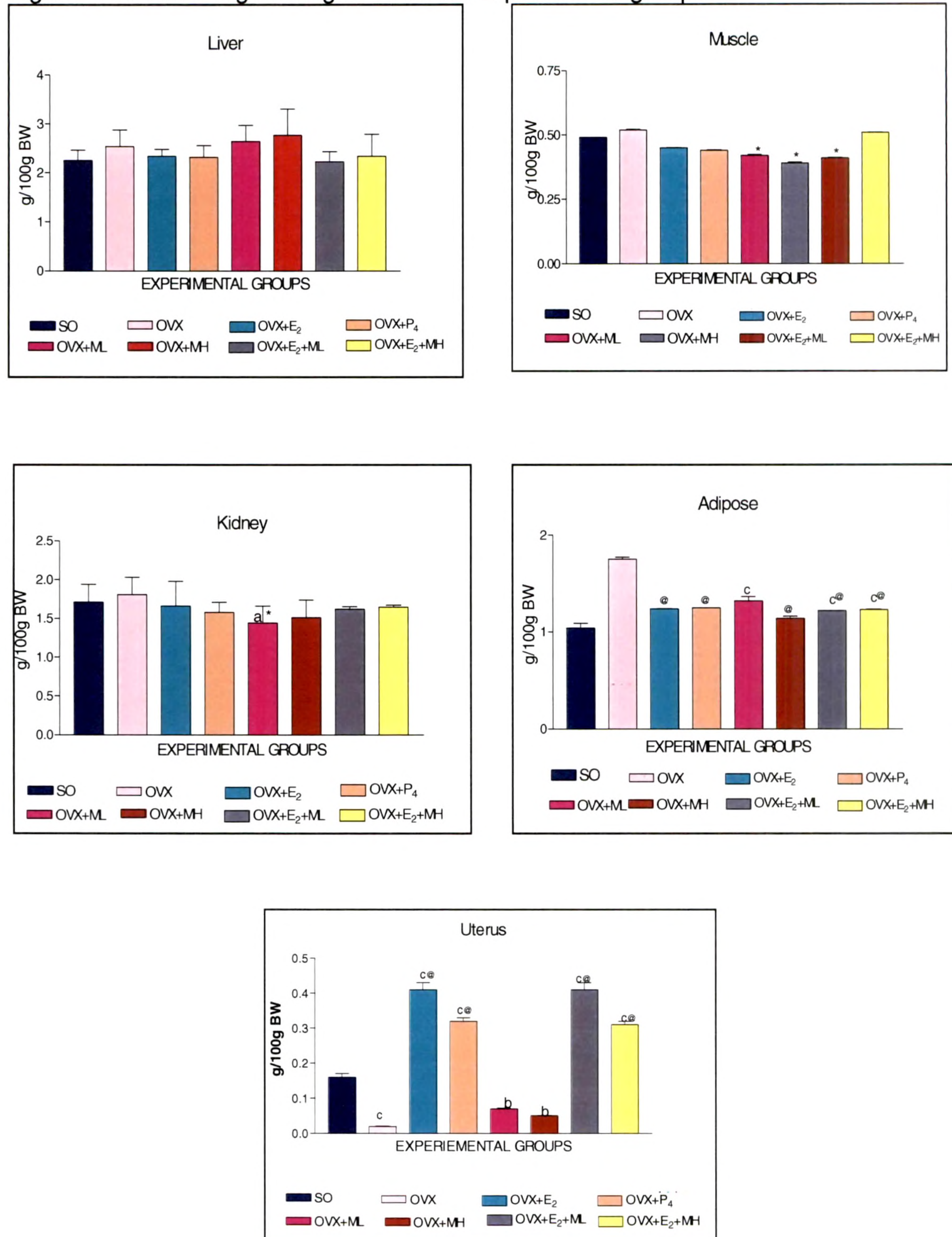
Data are expressed as Mean±SE
[b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [e]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Table 2 : Relative organ weights in all the experimental groups.

Relative weights (g/100g body weight)					
GROUPS	Liver	Muscle	Kidney	Uterus	Adipose
SO	2.25±0.21	0.49±0.001	1.71±0.54	0.16±0.01	1.04±0.05
OVX	2.54±0.34	0.52±0.002	1.81±0.36	0.02±0.001^{c}	1.75±0.023^{c}
OVX +E_2	2.34±0.14	0.45±0.0012	1.66±0.42	0.41±0.02$^{c@}$	1.24±0.003$^{@}$
OVX +P_4	2.32±0.24	0.44±0.0023	1.58±0.35	0.32±0.01$^{c@}$	1.25±0.0012$^{@}$
OVX +ML	2.64±0.33	0.42±0.0033*	1.44±0.23$^{c@}$	0.07±0.002^{b}	1.32±0.045^{c}
OVX +MH	2.77±0.54	0.39±0.0041*	1.51±0.27	0.05±0.001^{b}	1.14±0.023$^{@}$
OVX +E_2+ML	2.22±0.21	0.41±0.002*	1.62±0.035	0.41±0.02$^{c@}$	1.22±0.0024$^{c@}$
OVX +E_2+MH	2.34±0.45	0.51±0.0012	1.65±0.023	0.31±0.01$^{c@}$	1.23±0.0052$^{c@}$

Data are expressed as Mean±SE
$^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{@}p<0.001$ when compared to ovariectomized animals.

Figure 2: Relative organ weights in all the experimental groups.



Data are expressed as Mean±SE

[b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [@]p<0.001 when compared to ovariectomized animals.

Table 3: Fasting serum glucose and hormone profile in all the experimental groups.

(A)

GROUPS	Fasting Serum Glucose mM/l	INSULIN mU/l	FIRI
SO	5.24±0.12	7.51±0.25	1.574
OVX	4.77±0.11	12.52±0.32^{c}	2.39
OVX +E_2	5.37±0.13	8.26±0.24$^{c@}$	1.774
OVX +P_4	5.62±0.21	8.76±0.54$^{c@}$	1.97
OVX +ML	5.27±0.15	6.51±2.5$^{c@}$	1.37
OVX +MH	6.27±0.03^{be}	5.51±0.23$^{c@}$	1.38
OVX +E_2+ML	5.83±0.16^{a*}	5.51±0.12$^{c@}$	1.28
OVX +E_2+MH	6.46±0.05^{be}	5.26±1.20^{c*}	1.35

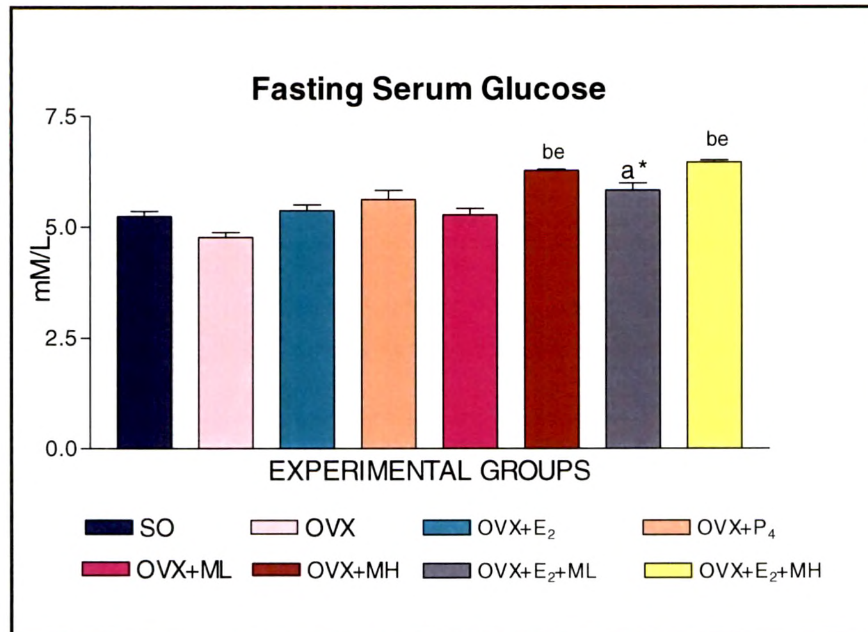
Data are expressed as Mean±SE
$^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{e}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

(B)

GROUPS	ESTROGEN pg/ml	PROGESTERONE ng/ml
SO	24.00±2.12	66.00±6.4
OVX	1.12±0.024^{c}	13.90±1.56^{c}
OVX +E_2	80.00±5.45$^{c@}$	10.10±2.11^{c*}
OVX +P_4	4.23±0.02	42.32±3.45$^{b@}$
OVX +ML	6.00±2.11^{b}	16.66±2.84^{c*}
OVX +MH	5.24±0.1^{c}	13.82±0.21^{c*}
OVX +E_2+ML	90.00±3.87$^{c@}$	24.23±5.85^{c}
OVX +E_2+MH	85.00±4.45$^{c@}$	32.00±3.58^{c*}

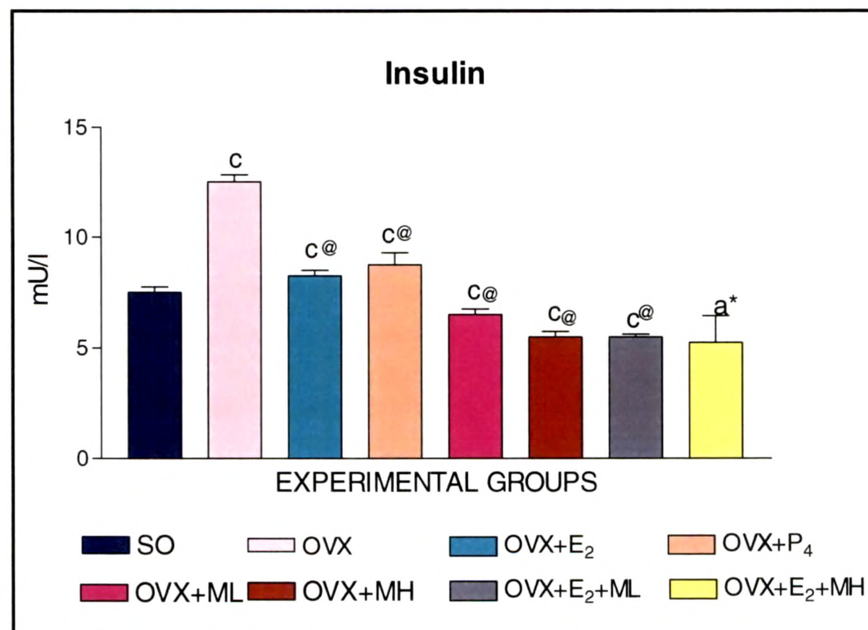
Data are expressed as Mean±SE
$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{@}p<0.001$ when compared to ovariectomized animals.

Figure 3.1: Fasting serum glucose in all the experimental groups.



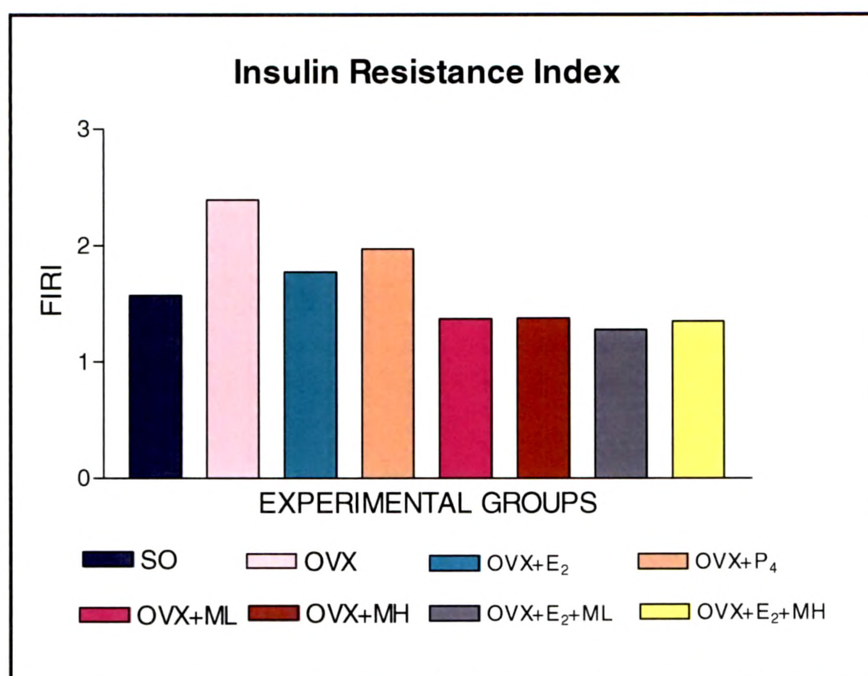
Data are expressed as Mean±SE
[b]$p<0.01$ when compared to sham operated control and [*]$p<0.05$, [e]$p<0.01$ when compared to ovariectomized animals.

Figure 3.2: Fasting insulin level in all the experimental groups.



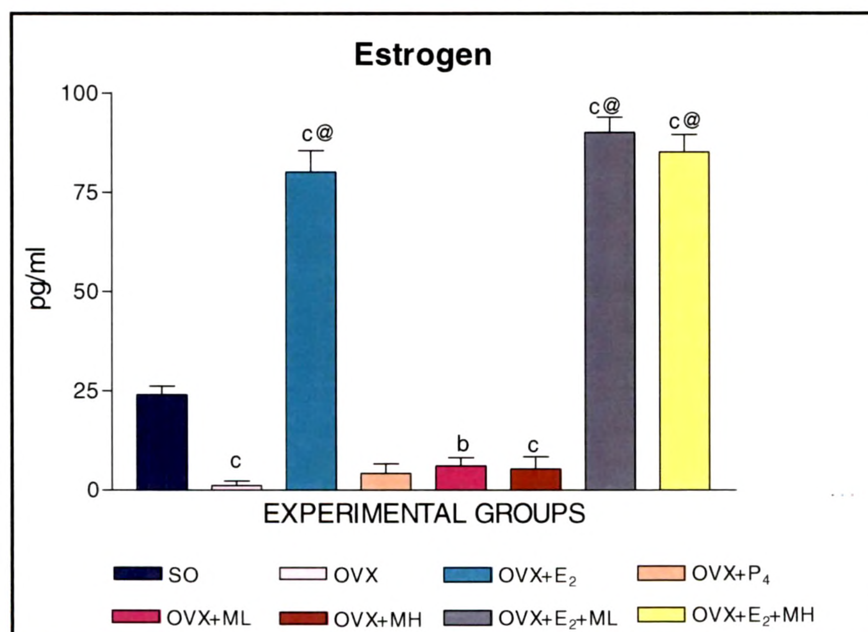
Data are expressed as Mean±SE
[a]$p<0.05$, [c]$p<0.001$ when compared to sham operated control and [*]$p<0.05$, [@]$p<0.001$ when compared to ovariectomized animals.

Figure 3.3 : Insulin sensitivity index in all the experimental groups.



SO= Sham operated control, OVX= Ovariectomized , OVX+E_2= Ovariectomized +Estrogen, OVX+P_4= Ovariectomized +Progesterone, OVX+ML= Ovariectomized+ Melatonin(Low dose), OVX+MH= Ovariectomized+ Melatonin(high dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(Low dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(High dose)

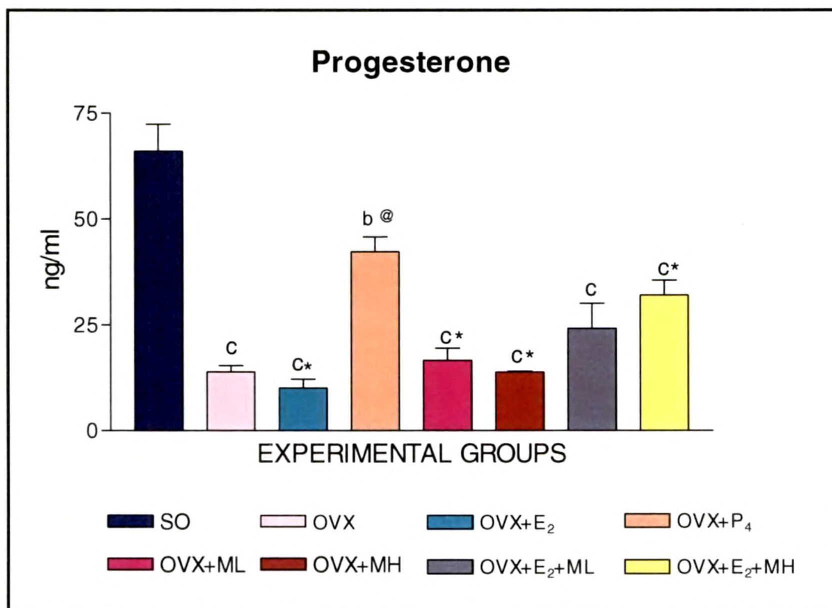
Figure 3.4 : Serum Estradiol level in all the experimental groups.



Data are expressed as Mean±SE

[b]$p<0.01$, [c]$p<0.001$ when compared to sham operated control and [@]$p<0.001$ when compared to ovariectomized animals.

Figure 3.5: Serum Progesterone level in all the experimental groups.



Data are expressed as Mean±SE
[b]$p<0.01$, [c]$p<0.001$ when compared to sham operated control and [*]$p<0.05$,[e]$p<0.01$, [@]$p<0.001$ when compared to ovariectomized animals.

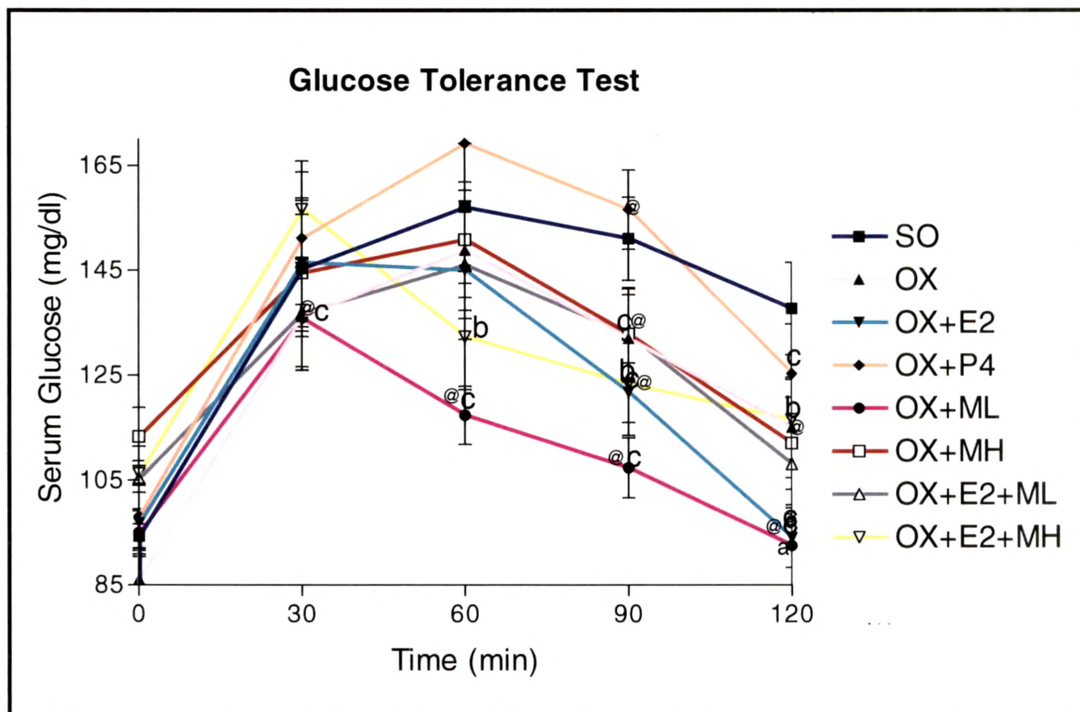
Table 4.1: Glucose tolerance curves of control and experimental rats.

GROUPS	0min	30min	60min	90min	120min
SO	94.33±2.32	145.33±13.02	167.00±12.21	151.00±7.96	137.66±8.78
OX	71.166±5.45	121.16±14.3	121.83±15.44	121.00±8.32	105.00±9.56
OX+E2	96.83±5.88	146.50±12.32	145.00±13.21	121.66±5.66 [c]	94.00±5.68 [c]
OX+P4	98.21±6.12	151.11±12.65	169.21±12.36	156.54±7.56 [@]	125.23±9.45
OX+ML	95.00±4.54	135.83±13.44 [a]	117.33±14.32 [c@]	107.33±9.68 [c @]	82.50±7.77 [c@]
OX+MH	113.33±5.51	136.50±11.11	130.83±11.02 [c]	112.83±8.88 [c]	96.00±8.79 [c]
OX+E2+ML	105.33±6.12	136.83±10.25	146.00±10.23	132.66±8.79 [c]	108.00±9.45 [c]
OX+E2+MH	86.33±2.35	146.66±10.23	123.33±13.13 [c]	116.33±9.87 [c]	106.50±7.65 [c]

Data are expressed as Mean±SE

[a]p<0.05, [c]p<0.001 when compared to sham operated control and [@]p<0.001 when compared to ovariectomized animals.

Figure 4.1: Glucose tolerance curves of control and experimental rats.



Data are expressed as Mean±SE

[b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [@]p<0.001 when compared to ovariectomized animals.

Table 4.2: Rates of elevation and clearance for the glucose tolerance curves

Elevation and Clearance rates for Glucose tolerance curves		
GROUPS	RATE OF ELEVATION	RATE OF CLEARANCE
SO	1.21	0.488
OX	0.83	0.266
OX+E2	1.65	0.583
OX+P4	1.83	0.730
OX+ML	1.33	0.588
OX+MH	0.77	0.450
OX+E2+ML	0.67	0.633
OX+E2+MH	2.01	0.446

SO= Sham operated control, OVX= Ovariectomized , OVX+E_2= Ovariectomized +Estrogen, OVX+P_4= Ovariectomized +Progesterone, OVX+ML= Ovariectomized+ Melatonin(Low dose), OVX+MH= Ovariectomized+ Melatonin(high dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(Low dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(High dose)

Figure 4.2: Area under curve for Glucose tolerance test in control and treated rats.

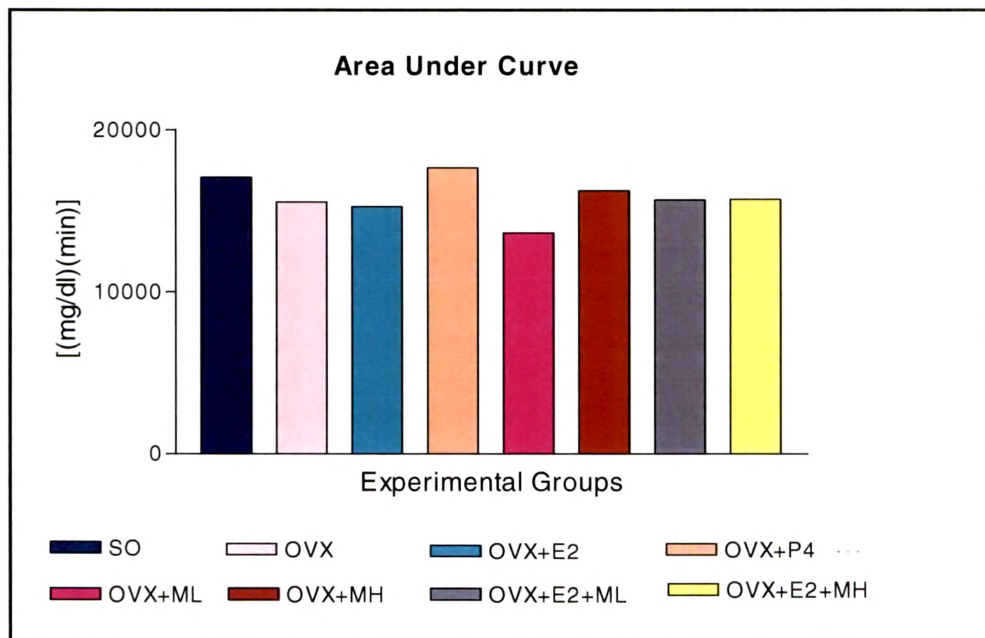


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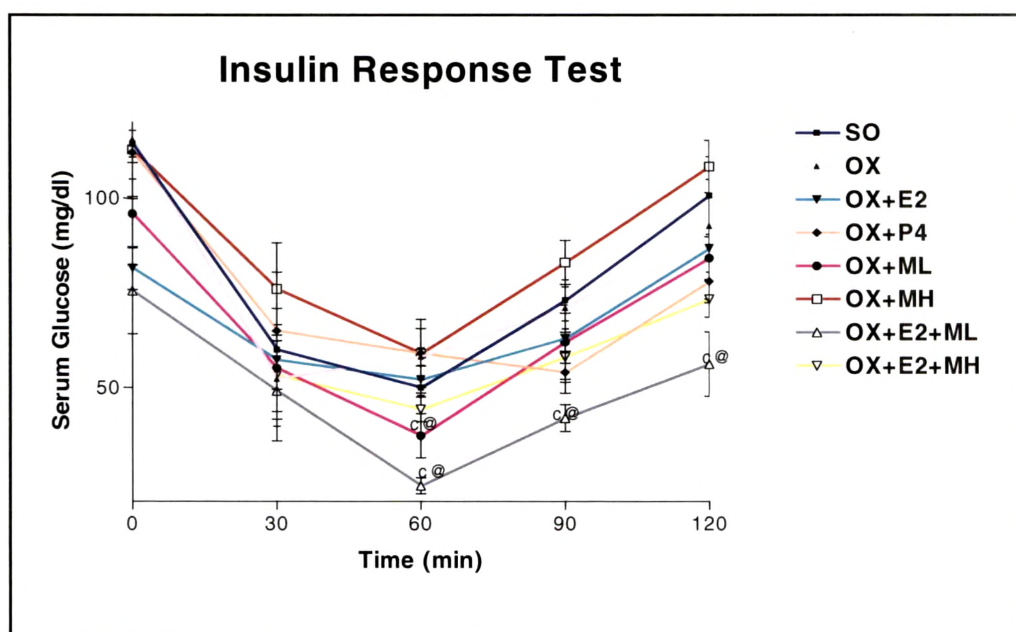
Table 5.1: Insulin response curves in control and experimental rats

GROUPS	0min	30min	60min	90min	120min
SO	114.33±3.54	50.00±2.31	50.00±2.11	73.00±5.45	100.66±10.21
OX	115.50±10.45	52.33±12.54	58.16±2.35	71.50±5.58	92.66±12.21
OX+E2	81.50±5.65	57.33±13.57	52.16±3.56	63.66±6.58	86.66±13.33
OX+P4	112.00±12.21	65.00±15.47	59.00±6.55	54.00±5.55	78.00±5.68 c@
OX+ML	95.83±13.54	55.16±11.45	55.33±5.77	62.33±9.78	84.16±5.66
OX+MH	112.83±12.45	56±12.14	69.00±8.99	83.66±5.85	108.33±6.87
OX+E2+ML	75.50±11.32 c@	49.16±13.25	24.16±2.11 c@	42.00±3.54 c@	56.16±8.45 c@
OX+E2+MH	123.66±15.41	44.33±11.63	53.33±3.21	58.66±6.58	73.33±4.75 c@

Data are expressed as Mean±SE
[c]p<0.001 when compared to sham operated control and [@]p<0.001 when compared to ovariectomized animals.

Figure 5.1: Insulin response curves in control and experimental rats



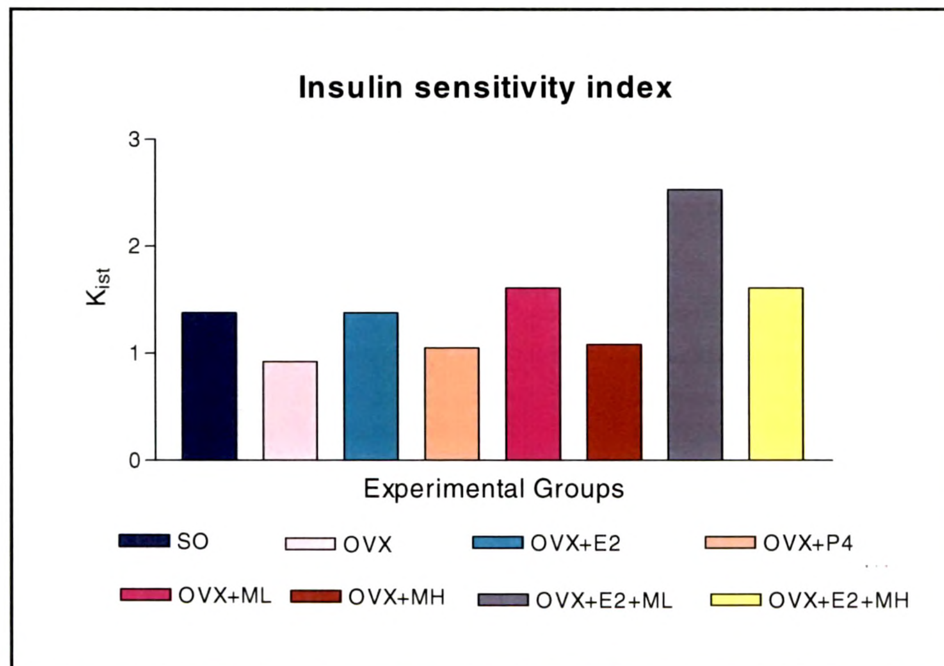
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [e]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Table 5.2: Rates of elevation and clearance for the glucose tolerance curves

Clearance and Elevation rates of Insulin response curves		
GROUPS	RATE OF CLEARENCE	RATE OF ELEVATION
SO	1.07	0.844
OX	2.1	1.220
OX+E2	0.80	0.575
OX+P4	0.64	0.800
OX+ML	0.68	0.319
OX+MH	1.89	0.580
OX+E2+ML	0.85	0.533
OX+E2+MH	2.64	0.322

SO= Sham operated control, OVX= Ovariectomized , OVX+E_2= Ovariectomized +Estrogen, OVX+P_4= Ovariectomized +Progesterone, OVX+ML= Ovariectomized+ Melatonin(Low dose), OVX+MH= Ovariectomized+ Melatonin(high dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(Low dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(High dose)

Figure 5.2 : Insulin sensitivity index in control and experimental rats.



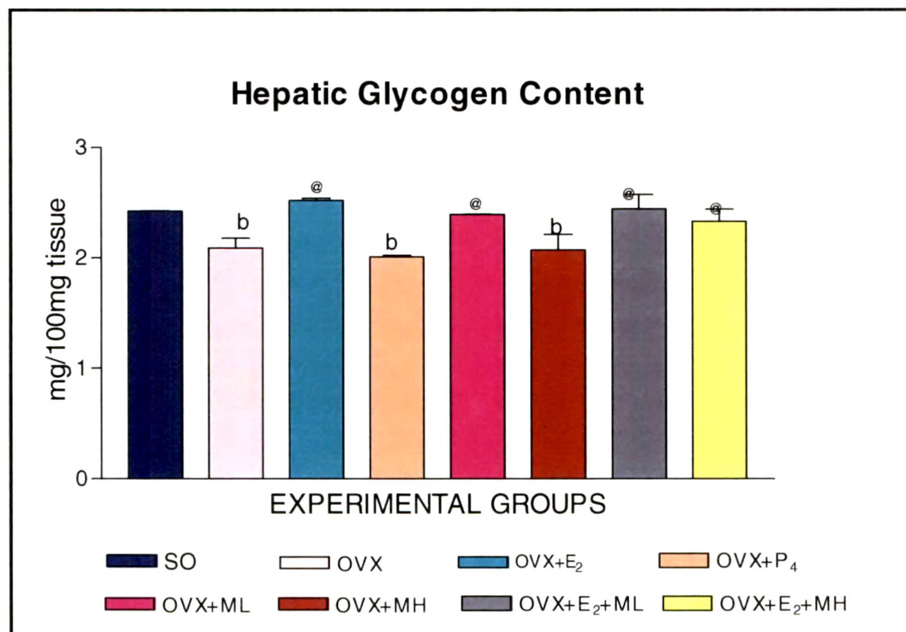
SO= Sham operated control, OVX= Ovariectomized , OVX+E_2= Ovariectomized +Estrogen, OVX+P_4= Ovariectomized +Progesterone, OVX+ML= Ovariectomized+ Melatonin(Low dose), OVX+MH= Ovariectomized+ Melatonin(high dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(Low dose), OVX+E_2+ML= Ovariectomized +Estrogen+ Melatonin(High dose)

Table 6: Hepatic Glycogen content and glycogen phosphorylase and G-6-Pase activity in control and experimental groups.

GROUPS	GLYCOGEN (mg/100mg tissue)	GLYCOGEN PHOSPHORYLASE (μM PO4 released /100mg protein/10min)	GLUCOSE 6 PHOSPHATASE (μM PO4 released /100mg protein/10min)
SO	2.42±0.006	0.128 ±0.001	0.23 ±0.0282
OVX	2.09±0.09 [b]	0.149±0.001 [c]	0.28 ±0.0110 [b]
OVX +E_2	2.52±0.02 [@]	0.125±0.0021 [@]	0.24±0.0021 [e]
OVX +P_4	2.01±0.012 [b]	0.144±0.0012 [c]	0.29±0.001 [b]
OVX +ML	2.39±0.004 [@]	0.131±0.0034 [@]	0.22 ±0.0341
OVX +MH	2.07±0.14 [b]	0.140±0.0001 [b]	0.26 ±0.0203
OVX +E_2+ML	2.44±0.13 [@]	0.120 ±0.001 [@]	0.23±0.0012 [e]
OVX +E_2+MH	2.33±0.11 [@]	0.135±0.0032 [@]	0.235±0.0022 [e]

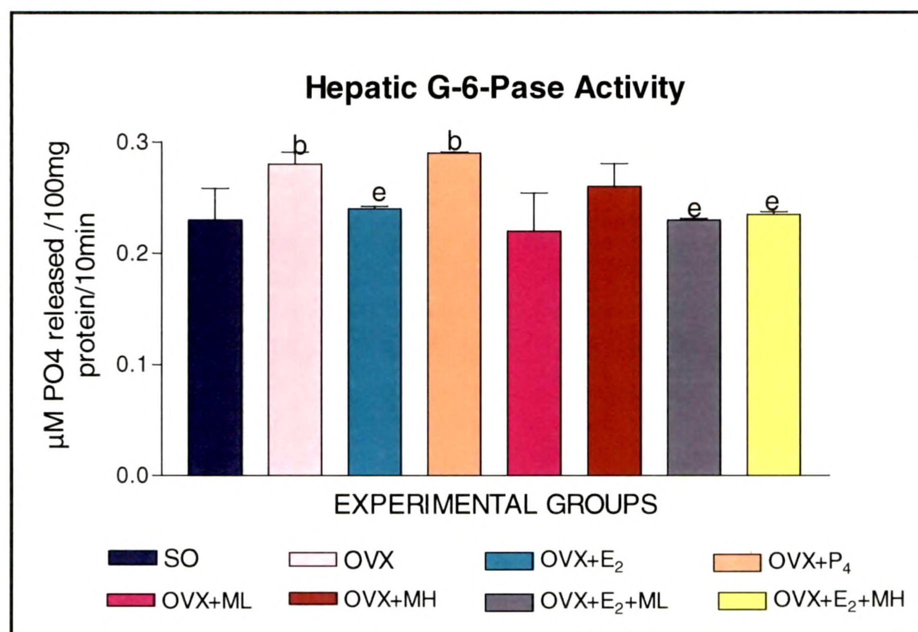
Data are expressed as Mean±SE
[b]$p<0.01$, [c]$p<0.001$ when compared to sham operated control and [e]$p<0.01$, [@]$p<0.001$ when compared to ovariectomized animals.

Figure 6.1: Hepatic Glycogen content in control and experimental groups.



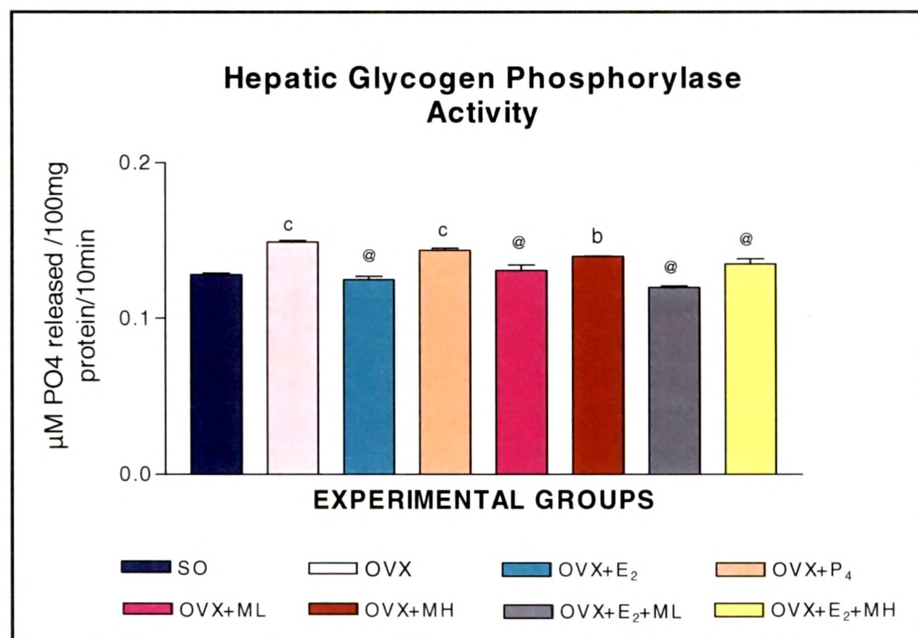
Data are expressed as Mean±SE
[b]$p<0.01$ when compared to sham operated control and [@]$p<0.001$ when compared to ovariectomized animals.

Figure 6.2: Hepatic glycogen phosphorylase in control and experimental groups.



Data are expressed as Mean±SE
[b]p<0.01 when compared to sham operated control and [e]p<0.01when compared to ovariectomized animals.

Figure 6.3: Hepatic G-6-Pase activity in control and experimental groups.



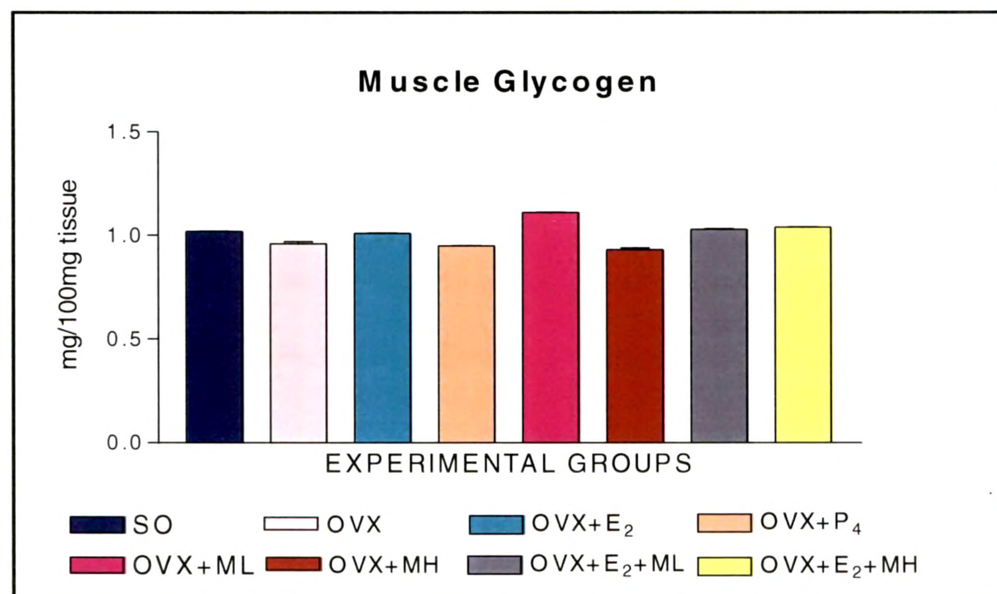
Data are expressed as Mean±SE
[b]p<0.01, [c]p<0.001 when compared to sham operated control and [@]p<0.001 when compared to ovariectomized animals.

Table 7 : Changes in muscle glycogen contents and phosphorylase activity in control and experimental animals.

GROUPS	GLYCOGEN (mg/100mg tissue)	GLYCOGEN PHOSPHORYLASE (μM PO4 released /100mg protein/10min)
SO	1.02±0.001	0.25 ±0.003
OVX	0.96±0.01	0.28 ±0.001
OVX +E_2	1.01±0.002	0.24±0.001 [e]
OVX +P_4	0.95±0.0012	0.29±0.002 [b]
OVX +ML	1.11±0.0014	0.23 ±0.010[e]
OVX +MH	0.93±0.01	0.27 ±0.001
OVX +E_2+ML	1.03±0.002	0.25±0.023
OVX +E_2+MH	1.04±0.01	0.27±0.001

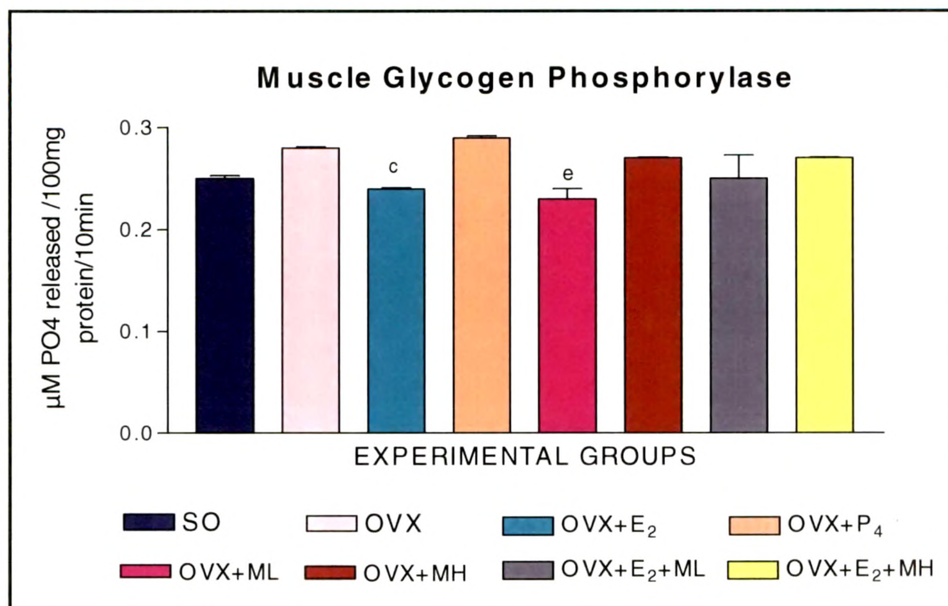
Data are expressed as Mean±SE
[b]p<0.01when compared to sham operated control and [e]p<0.01when compared to ovariectomized animals.

Figure 7.1: Changes in muscle glycogen contents in control and experimental animals.



Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [e]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure 7.2: Changes in muscle phosphorylase activity in control and experimental animals.



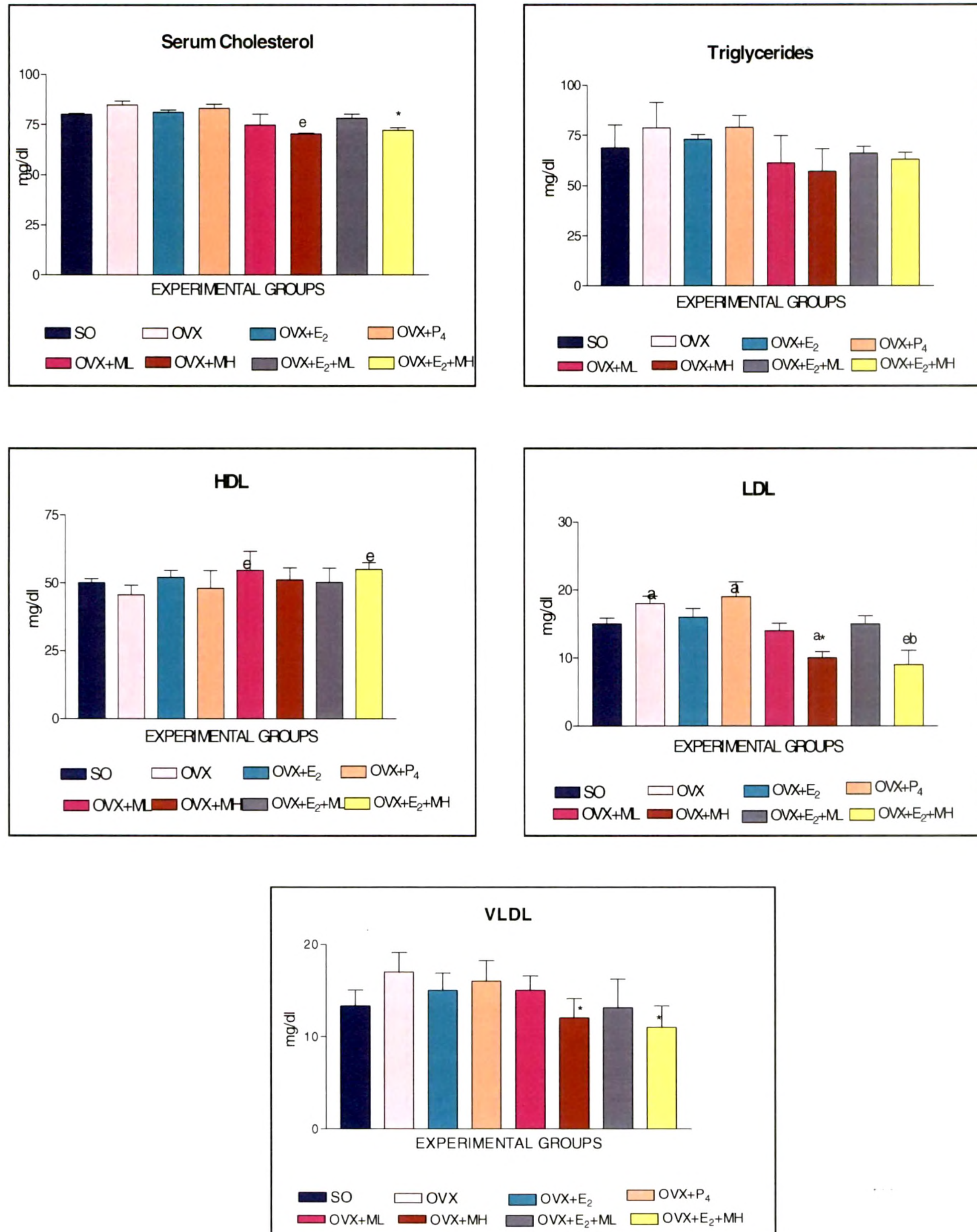
Data are expressed as Mean±SE
[c]p<0.001 when compared to sham operated control and [e]p<0.01when compared to ovariectomized animals.

Table 8: Changes in serum lipid profile in control and experimental groups

SERUM LIPID PROFILE: (mg/dl)					
GROUPS	CHO	TG	HDL	LDL	VLDL
SO	80.00 ±2.58	68.67 ±11.42	50.00 ±1.53	15.00±1.87	13.33±1.73
OVX	84.67 ±2.03	78.67 ±12.73	45.67 ±3.48	18.00±1.12[a]	17.00±2.11
OVX +E_2	81.12±1.21	73.00±2.35	52.00±2.65	16.00±1.32	15.00±1.87
OVX +P_4	83.00±2.11	79.00±5.88	48.00±6.54	19.00±2.22[a]	16.00±2.24
OVX +ML	74.67 ±5.46	61.33 ±13.58	54.67 ±6.90[e]	14.00±1.11	15.00±1.58
OVX +MH	70.33 ±3.33[e]	57.00 ±11.28	51.00±4.58	10.0 ±2.94[a*]	12.00±2.1[*]
OVX +E_2+ML	78.00±2.22	66.00±10.21	50.22±5.25	15.00±1.21	13.12±3.12
OVX +E_2+MH	72.00±1.34[*]	63.00±3.45	55.00±2.55[e]	9.00±2.14[eb]	11.00±2.3[*]

Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01when compared to sham operated control and [*]p<0.05, [e]p<0.01when compared to ovariectomized animals.

Figure 8: Changes in serum lipid profile in control and experimental groups



Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [e]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Table 9: Tissue lipid and cholesterol contents in control and experimental groups

(A)

Cholesterol (mg/100mg tissue)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	0.32 ±0.04	0.13 ±0.04	0.40 ±0.02
OVX	0.44 ±0.018	0.16 ±0.02	0.61 ±0.04[c]
OVX +E_2	0.39±0.05	0.11±0.002[*]	0.55±0.058[b]
OVX +P_4	0.42±0.065	0.17±0.031	0.59±0.033[b]
OVX +ML	0.42 ±0.08	0.12 ±0.015	0.51 ±0.05[b]
OVX +MH	0.32 ±0.04[e]	0.10 ±0.05	0.42 ±0.047[@]
OVX +E_2+ML	0.38±0.021	0.12±0.012	0.52±0.052[ea]
OVX +E_2+MH	0.29±0.024[e]	0.10±0.032	0.51±0.053[ea]

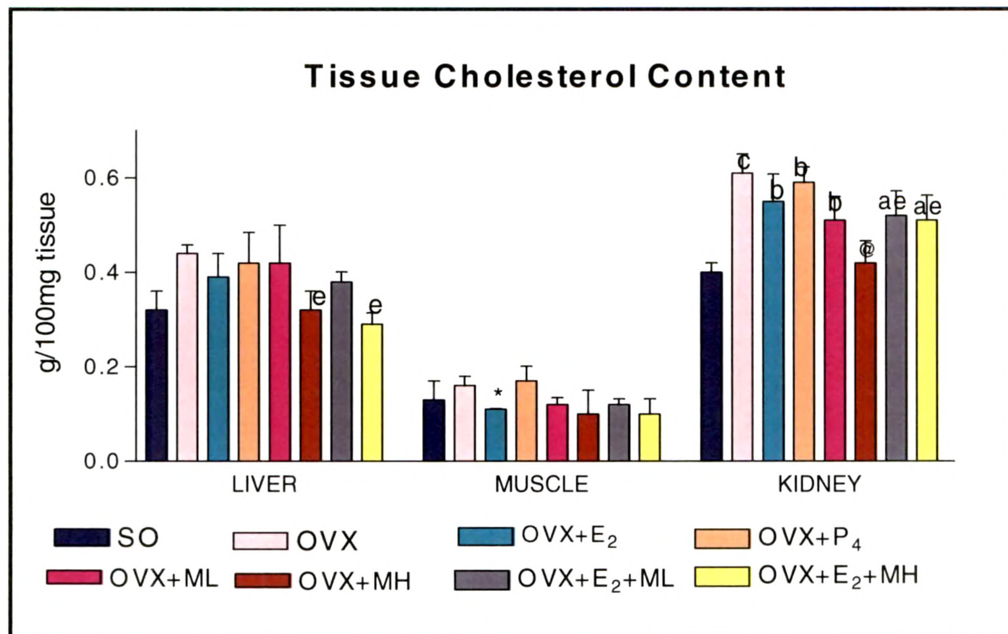
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [e]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

(B)

LIPID(mg/100mg tissue)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	4.01±0.071	1.86±0.043	0.85±0.06
OVX	4.85±0.054[b]	1.95±0.045	0.88±0.054
OVX +E_2	4.21±0.056[e]	1.91±0.055	0.84±0.057
OVX +P_4	4.65±0.068[b]	1.93±0.065	0.87±0.061
OVX +ML	4.20±0.065[e]	1.87±0.023	0.82±0.047
OVX +MH	4.02±0.057[e]	1.78±0.061	0.76±0.062
OVX +E_2+ML	4.30±0.085[e]	1.89±0.078	0.85±0.088
OVX +E_2+MH	4.02±0.098[e]	1.75±0.044	0.80±0.021

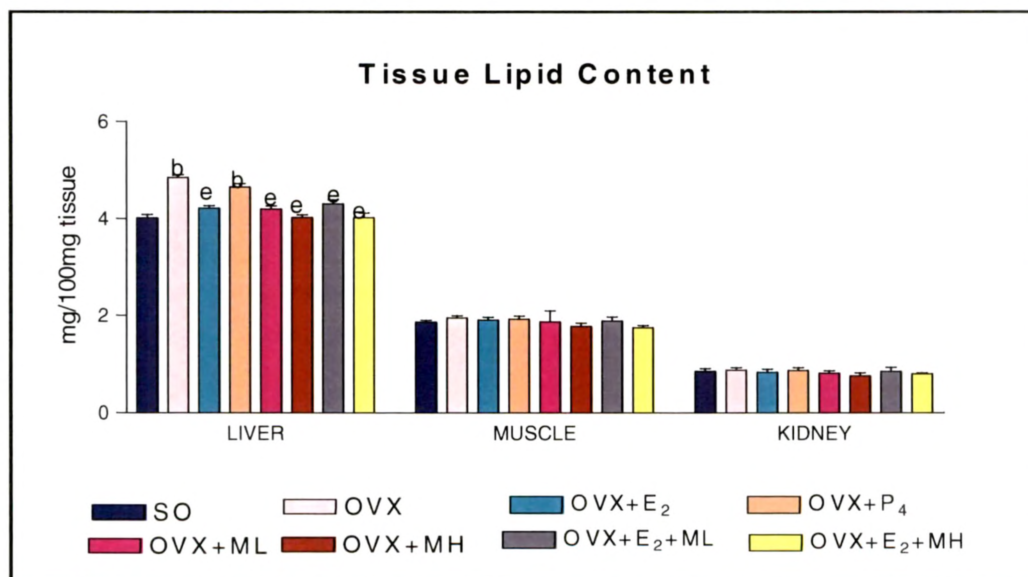
Data are expressed as Mean±SE
[b]p<0.01when compared to sham operated control and [e]p<0.01when compared to ovariectomized animals.

Figure 9.1: Tissue lipid content in control and experimental groups



Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [e]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure 9.2: Tissue cholesterol contents in control and experimental groups



Data are expressed as Mean±SE
[b]p<0.01when compared to sham operated control and [e]p<0.01when compared to ovariectomized animals.

DISCUSSION

The present study employs ovariectomized rats as an experimental model to study the changes associated with menopause as it is characterized by mild obesity and thus helps to understand the changes in adiposity associated with estrogen deficient state (Wade *et al.*, 1985). Efficacy of melatonin as a replacement therapy has been treated as, ovariectomized rats that are known to exhibit characteristics of increased visceral fat content, dyslipidemia, impaired glucose tolerance and defective insulin mediated glucose as seen in insulin resistance (Saengsirisuwan *et al.*, 2009). The study evaluates the potentials of melatonin in reversing insulin resistance and other diabetogenic changes associated with menopause based on the analysis of parameters related to carbohydrate and lipid metabolism as compared to the merits and demerits associated with the class of ERT and P_4 replacement. Based on our previous studies (Singh, 2010; Singh *et al.*, 2010a, b), two different doses of melatonin have been employed in the present study to determine the dose dependent response in terms of Melatonin supplementation test (MST).

Ovariectomized rats in the present evaluation are characterized by an increased body weight gain and feed efficiency due to increased food intake compared to sham operated controls. MST could effectively regulate ovariectomy induced weight gain as well as feed efficiency. These changes are more pronouncedly seen in the ML treated OVX rats than in either Estrogen or progesterone supplemented rats. Though previous findings do establish the role of ERT in reversing the increased weight gain and

controlling energy expenditure and obesity associated with menopause, our results indicate that melatonin has the most potent role in preventing body weight gain than estrogen alone or estrogen - melatonin combination. Our observations are at variance with the findings of Sanchez-Mateos *et al.* (2007) wherein, melatonin administered through drinking water had intermediate effect in reducing body weight gain as compared to estrogen or E_2+M supplemented groups. The difference between the two observations may be attributed to the disparity in the mode of melatonin supplementation in the two studies. Our schedule of evening Melatonin administration is in keeping with the natural Melatonin rhythm, known to be maximally effective in terms of favourable actions of ML (Maestroni *et al.*, 1987; Krauchi *et al.*, 1997; Daniel *et al.*, 2006) as against continuous Melatonin intake through drinking water likely to have minimal effect. The increased body weight gain seen in OVX animals is also associated with increased adiposity, which is also nullified by MH as well as E_2+MH and E_2+ML supplementation even better than in E_2 replacement or ML supplementation. The OVX animals also show a significant reduction in uterine weight owing to the removal of the circulating sex steroids which is fully reversed in E_2 and P_4 replaced groups in that order as also shown by other studies (Wang *et al.*, 2004; Saengsirisuwan *et al.*, 2009). Though E_2+ML and E_2+MH were also equally effective, MST was however ineffective. This ineffectiveness of MST in recovering uterine weight is however of no consequence or significance in menopausal/post menopausal women.

The increase in serum titre and increased FIRI index seen in the present study are well supported by the reported whole body insulin resistance and elevated insulin level in rats after 5 weeks of ovariectomy (Lui *et al.*, 2004). Melatonin administration in the present study was successful in bringing about significant reduction in serum insulin level and maintaining fasting glucose level alongwith bettered FIRI index. Our study on MST employing two doses of M given alone or in combination with E_2 has revealed the former to be maximally effective than the latter (combination) in maintaining glycaemic status, serum insulin titre and FIRI; in fact both these schedules proved to be better than ERT. The present report is the foremost and the only one of its type to show efficacy of M in controlling insulin resistance and fasting insulin and glucose levels in OVX animals and hence providing adequate support for MST as a possible alternative to ERT in post menopausal women. Melatonin has been shown to reduce the circulating insulin levels as evidenced from our previous studies that provide further basis to the present contention (Singh *et al.*, 2010a) as also other studies (Marzena *et al.*, 2002; Stumpf *et al.*,2008).

Both GTT and IRT have shown significantly increased area under curve for the former and significant reduction in K_{IST} for the latter in OVX animals. In another form of evaluation on insulin sensitivity expressed as Glucose-insulin (G-I) index based on time dependent changes in glucose and insulin levels under GTT, has also reported decreased insulin sensitivity marked by higher G-I index in OVX rats (Saengsirisuwan *et al.*, 2009). Impaired glucose tolerance and decreased insulin sensitivity have also been

demonstrated by others in OVX animals (Kumagai *et al.*, 1993; Rincon *et al.*, 1996). In the present study, neither ERT nor PRT has been potent in reversing the OVX induced impairment in glucose tolerance and this finds relevance from the reports of worsened glucose tolerance with low or high dose oral contraceptive use (Godsland *et al.*, 1990; Wynn and Doar, 1966;) or even in post menopausal HRT depending on dose and type of steroid used (Godsland *et al.*, 1996). However, the response to exogenous insulin in insulin response test does show a significant degree of normalization in ERT rats not observable in PRT is also evidenced by (Kumagai *et al.*, 1993) where, estrogen treatment restored insulin sensitivity and progesterone treatment resulted in insulin resistance. Melatonin administration either alone or in combination with estrogen did register better glycaemic regulation by improving glucose tolerance with lesser area under curve and, improving insulin sensitivity as seen from the K_{IS} value. Of all the M supplementation groups OVX+E_2+ML has shown the most effective response in terms of both glucose tolerance and insulin sensitivity with OVX+ML, OVX+E_2+MH and OVX+ E_2 being the next in their order of efficacy. In this context, the effectiveness of melatonin in maintaining the glucose homeostasis in diabetic and non diabetic animals has been reported from our laboratory and elsewhere (Peschke, 2008; Peschke *et al.*, 2008; Singh *et al.*, 2010a, b). Apparently, the present findings highlight the fact that MST with a low dose is much more effective than ERT in impairing insulin sensitivity, though MST in combination with E_2 had the maximal effects.

Impaired carbohydrate metabolism in OVX animals is indicated by decreased hepatic and muscle glycogen contents with concomitant increased phosphorylase activity and elevated hepatic Glucose-6-phosphatase activity, changes which can be considered to be diabetogenic (Sun *et al.*, 2004). Ovariectomized animals as a model for mild obesity and insulin resistance are characterized by reduced glycogen contents (Sun *et al.*, 2004) and impaired activation of insulin stimulated glycogen synthesis in insulin resistant states has also been reported (Yeaman *et al.*, 2001). Estrogen but not progesterone is seen to be effective in reversing the OVX induced changes in terms of tissue glycogen content and activities of phosphorylase and G-6-Pase. In animals subjected to MST, ML was as effective as ERT or even a combination of MST+ERT in combating ovariectomy induced alterations in carbohydrate metabolism, which is well supported by our studies on diabetic animals (unpublished data).

A mechanistic explanation for the observed decrease in tissue glycogen content and insulin resistance could be ovariectomy induced reduction in GLUT 4 expression in peripheral tissues and consequent compromised glucose uptake. Evidence to this end is available from the works of Saengsirisuwan *et al.* (2009) showing reduced muscle GLUT 4 expression OVX animals and its recovery upon E_2 replacement and of Barros *et al.* (2006) demonstrating insulin resistance by way of GLUT-4 under expression in Estrogen receptor knockout mice (ERα $^{-/-}$). Our demonstration herein of reduced insulin resistance in OVX rats subjected to MST is well supported by the observations from studies in our laboratory showing the

ability of melatonin to increase GLUT-4 expression in GLUT-4 deficient diabetic rats (Singh, 2010). Apparently, MST is competent enough to restore glycaemic dysregulation, insulin resistance and reduced glucose uptake brought about by estrogen deficiency and could be thought as an alternative to E_2.

Alongwith impairment in carbohydrate metabolism, dysregulation of lipid metabolism is also indicated by hyperlipidemia and hypercholesterolemia and increased tissue lipid and cholesterol load, very much in tune with the noted increased adiposity. Even other workers have noted such changes in OVX rats used as model for understanding post-menopausal changes (Wade *et al.*, 1985; Saengsirisuwan *et al* 2009; Picard *et al.*, 2000) of the various replacement/supplementation therapies in the present study, MH and E_2+MH seemed to be maximally effective in rectifying the OVX induced lipidemia, cholesterolemic and adiposity follows by ML, E_2+ML and E_2 in that order. It is interesting to note that MST with ML was equally effective or even better than ERT in correcting ovariectomy induced dyslipidemia and tissue lipid load. This observation is in disparity with the report of no significant effect of M in OVX rats (Sanchez-Mateos *et al.*, 2007). Melatonin being a tricky hormone requires an appropriate means of administration in terms of dose, time and duration. The difference in the mode of administration (evening v/s continuous) as discussed earlier seems to best explain the observed discrepancies. Antihyperlipidemic and anti-hypercholesterolemic effects of melatonin have been well documented in both experimental animals and in humans (Wakatsuki *et al.*, 2001; Sener *et al.*, 2004). Present findings are further

embellished by our previous studies consistent with melatonin mediated regulation of dyslipidemia (Singh *et al.*, 2010a,b).

In conclusion, our data suggest a dose dependent efficacy of melatonin as a supplementation therapy in controlling ovariectomy induced insulin resistance and dyslipidemia. This study clearly indicates MST to be more potent and effective in comparison to ERT as it is able to revert the ovariectomy induced changes single handedly except for uterine weight and thus can compensate for sex steroid deficiency without any attendant side effects. An intermediate dose, in-between the two doses employed in the present study may be ideal in combating all ovariectomy /post-menopausal changes in keeping with the observed differential effects on carbohydrate and lipid metabolisms.