DIABETIC GLUCOSE DYSHOMEOSTASIS AND DYSLIPIDEMIA IN ESTROGEN DEFICIENT RATS: MELATONIN SUPPLEMENTATION MORE POTENT THAN ERT IN ALLEVIATING THE SYMPTOMS

Post menopausal women with diabetes mellitus is a relatively well established fact (Wedisinghe and Perera, 2009) and, is a major predisposing factor towards cardiovascular diabetes and leading to increasing morbidity and death in women (Dept of health, London. 2003). Menopause is marked by very little change in overall circulating insulin level; nevertheless there is increasing insulin resistance with age, predisposing women in the postmenopausal period towards development of type II diabetes (Wedisinghe and Perera, 2009). Care of postmenopausal women with diabetes mellitus is therefore one of the serious clinical issues of concern in the background of high prevalence of diabetes in aging population. Apart from natural menopause, there are also increasing cases of premature ovarian failure (POF) or surgical menopause in middle aged and younger women who are also likely to suffer from menopause related discomforts and also be predisposed to cardiovascular complications, bone defects, diabetes etc (Dorman *et al.*, 2001; Wedisinghe and Perera, 2009; Shuster *et al.*, 2010).

Hormone replacement therapy (HRT) is used widely to overcome the basic symptoms associated with menopause. In cases of POF and surgical/medical menopause, lifelong ERT becomes a need to maintain their normal physiology and health. However, use of HRT requires stringent regulation owing to the increased risk of cancer (upto 8 folds) in women on

prolonged unopposed HRT (Grady *et al.*, 1995). Moreso, with reference to the effect of HRT on carbohydrate metabolism, there are reports advocating use of low dose estrogen therapy as against high dose as, it has been shown to have detrimental effects on insulin sensitivity (Cagnacci *et al.*, 1992). Further, postmenopausal diabetes poses an added complication in HRT programs as; HRT is prescribed less commonly in women with diabetes owing to its disputed status (Feher and Isaacs, 1996; Lawrenson *et al.*, 1998; Bonds *et al.*, 2006). This necessitates search for alternatives to HRT that could effectively target both menopausal symptoms and diabetic complications.

Melatonin, earlier believed to have an important role only in maintaining circadian rhythm and reproductive physiology (Simonneaux and Ribelayga, 2003; Hazlerigg and Loudon, 2008; Nakao *et al.*, 2008; Reiter *et al.*, 2009) has been extensively studied and is reported to play important role in many other physiological functions. It has the ability to act as a potent antioxidant, being a powerful neutralizer of hydroxyl radical (Tan *et al.*, 2001). There are evidences of its beneficial glucoregulatory roles in ameliorating diabetic manifestations (Paskaloglu *et al.*, 2004; Peschke, 2008). Long term melatonin administration has been reported to reduce hyperlipidemia, hyperinsulinemia and restore ratios of PUFA in serum and tissues of diabetic rats (Nishida, 2005).

In view of the above cited roles and in combination of our earlier study detailing with the beneficial and more potent role of melatonin in alleviating various parameters of glycaemic regulation and dyslipidemia, the present study was designed to evaluate the efficacy of melatonin singly or in

combination with estrogen as a replacement therapy in ovariectomized diabetic rats. This study is the only one of its kind that is designed to study melatonin supplementation as an alternative therapy (MST) in post menopausal subjects with diabetes.

RESULTS:

Body weight gain and food intake and water intake (Table 1, Fig 1)

Table 1 shows the changes in food and water intake and body weight gain at the end of 5 weeks of study in control and experimental rats. Ovary intact and OVX diabetic rats showed significant increment in water and food intake with reduced body weight gain. Both ERT and MST significantly decreased the diabetes induced increases in food and water intakes. Body weight gain was also recovered to the control range with ERT and MST. However, MST_L and MST_L+ERT combination in that order depicted the most significant recovery in these parameters while, P_4 had effects similar to that ERT.

Relative organ weights (Table 2, Fig 2)

Table 2 depicts the relative organ weights of all the experimental groups. Untreated ovary intact and OVX diabetic animals showed significant increase in the relative weights of liver and muscle and decrease in kidney weight. Supplementation with M_H showed significant reduction in the relative weighs of all the three organs. Combination of E_2+MH in OVX diabetic rats was next best in terms of recovery. Relative weight of uterus increased significantly in ovary intact diabetic rats while, it was decreased in OVX diabetic rats.

Estrogen replacement increased the uterine weight in OVX diabetic rats. Whereas MST_L and MST_H had no significant effect, the uterine weight was increased to some extent in P_4 supplemented and MST_L+ERT as well as MST_H+ERT groups of rats.

Serum levels of glucose, insulin, E_2 and P_4 (Table 3, Figs 3A, 3B, 3C, 3D, 3E)

Table 3 shows the serum levels of glucose, insulin, E_2 and P_4. There was significant fasting hyperglycaemia in ovary intact and OVX diabetic rats compared to sham operated and OVX rats. The level of glucose was however relatively lower in OVX+D rats compared to D rats. Supplementation with M_L showed significant anti-hyperglycaemic effect comparable to ERT rats. There was marked decrement in serum insulin titre in D and OVX+D rats.

There was significant increment in the circulating E_2 level in D animals while, OVX+D animals did not show any variation in their E_2 levels. The E_2 titre was increased to a greater extent in all the ERT groups while, those supplemented with either melatonin or P_4 alone did not show any significant increment in E_2 level. On the other hand, P_4 level decreased in D animals compared to sham operated control animals and, the decrease was even greater in OVX+D animals when compared to OVX animals.

Oral glucose tolerance, Insulin response tests and Insulin sensitivity index (Tables 4, 5 and Figs 4, 4A, 5, 5A)

Figures 4 and 4A depict GTT and AUC in relation to GTT. The curves and AUC revealed significant deterioration in glucose tolerance in diabetic rats (D and OVX+D rats). Supplementation with M_L significantly decreased the AUC

of these rats with a bettered glucose tolerance curve in comparison to OVX+D rats. Even ERT, P_4 supplementation and MST_L with E_2 were also equally effective in reducing AUC.

Figures 5 and 5A depict the insulin response curves and insulin sensitivity index respectively of all the experimental groups. Untreated diabetic animals in both ovary intact and OVX rats showed significantly lowered insulin sensitivity as compared to their respective control groups with lower K_{IS} values. Ovariectomized diabetic rats subjected to MST_L showed bettered insulin response curve and with a higher insulin sensitivity index value.

Carbohydrate metabolism (Tables 6, 7 and Figs 6A, 6B, 6C, 7A, 7B)

Changes in hepatic glycogen content and activities of glycogen phosphorylase and Glucose-6-phosphatase depicted in table 6. Both D and OVX+D groups of animals showed significant decrement in tissue glycogen content and increase in the activity levels of both the enzymes in comparison to SO and OVX group of animals. Both MST and ERT alone or in combination have shown a trend of reversal and increase in hepatic glycogen content from the low level in OVX+D animals in the order OVX+D+ML> OVX+D+E_2+ML > OVX+D+E_2+MH >OVX+D+MH. Progesterone replacement was of no significant consequence. With respect to the enzyme activities, MSTs and their combination with E_2 only showed significant decrement in the order MST_L>MST_H>MST_L+ERT>MST_H+ERT>ERT>P_4.

Table 7 shows changes in glycogen content and glycogen phosphorylase activity in muscle. Diabetes significantly increased glycogen phosphorylase activity and decrease glycogen content in both D and OVX+D animals. Whereas, OVX+D+M_L and OVX+D+E_2+ML in that order showed maximal increment in glycogen content and decrement in phosphorylase activity, all other groups showed lesser but similar difference of changes.

Serum lipid profile (Table 8 and Fig 8)

Table 8 depicts the changes in serum lipid profile of all the experimental groups. Both ovary intact and OVX diabetic animals showed significantly increased levels of CHO, TG, LDL and VLDL and decrease and level of HDL. Supplementation with both doses of melatonin individually could significantly revert back the diabetes induced changes in CHO, TG, LDL, VLDL and HDL. Out of the two combinational treatments used OVX+D+E_2+M_L was better and all other schedules (ERT, P_4 and ERT+MST_H) were of lesser and almost equal effect.

Tissue cholesterol and lipid contents (Liver, Muscle and Kidney) (Table 9 and Fig 9)

Table 9 shows the tissue cholesterol and lipid contents of all the experimental groups. There was a significant increase in the total tissue cholesterol and lipid contents of all the three tissues in both D and OVX+D group of animals. Melatonin at a high dose singly and in combination to estrogen could effectively decrease the diabetes induced increase in tissue cholesterol while

E_2 and P_4 treatments proved to be similar and less significant in decreasing the tissue cholesterol content of all the three tissues. OVX+D+MH and OVX+D+E_2+MLshowed most significant decrement in tissue lipids while rest of the groups were effective in the order OVX+D+ML>OVX+D+E_2+MH>OVX+E_2 and P_4did not show any significant changes in the tissue lipid content.

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

GROUPS	Food Intake (g/animal/day)	Water Intake (ml/animal/day)	Body Weight Gain (g)
SO	17.55±1.03	36.12±5.12	10.00 ± 0.11^{c}
OVX	28.23 ± 1.44^{b}	40.32±4.45	28.00 ± 0.22^{c}
D	29.00 ± 2.23^{b}	76.56 ± 3.31^{c}	$3.32\pm0.33^{@}$
OVX+D	35.13 ± 2.31^{b}	$73.50\pm5.33^{c@}$	$5.00\pm0.42^{@}$
OVX+D+E_2	29.00±4.12	66.00 ± 5.26^{ce}	$11.00\pm0.33^{@}$
OVX+ D+P_4	30.00 ± 3.54^{b}	$70.00\pm3.22^{c@}$	$10.00\pm0.48^{@}$
OVX+ D+ML	24.00±1.95	45.00±4.67	$13.00\pm0.15^{c@}$
OVX+ D+MH	29.00 ± 2.44^{b}	56.00±2.54	$14.00\pm0.14^{c@}$
OVX+ D+E_2+ML	25.00±1.03	48.00±3.66	$16.00\pm0.19^{c@}$
OVX+ D+E_2+MH	26.00 ± 4.02^{b}	60.00 ± 3.12^{b}	$15.00\pm0.01^{c@}$

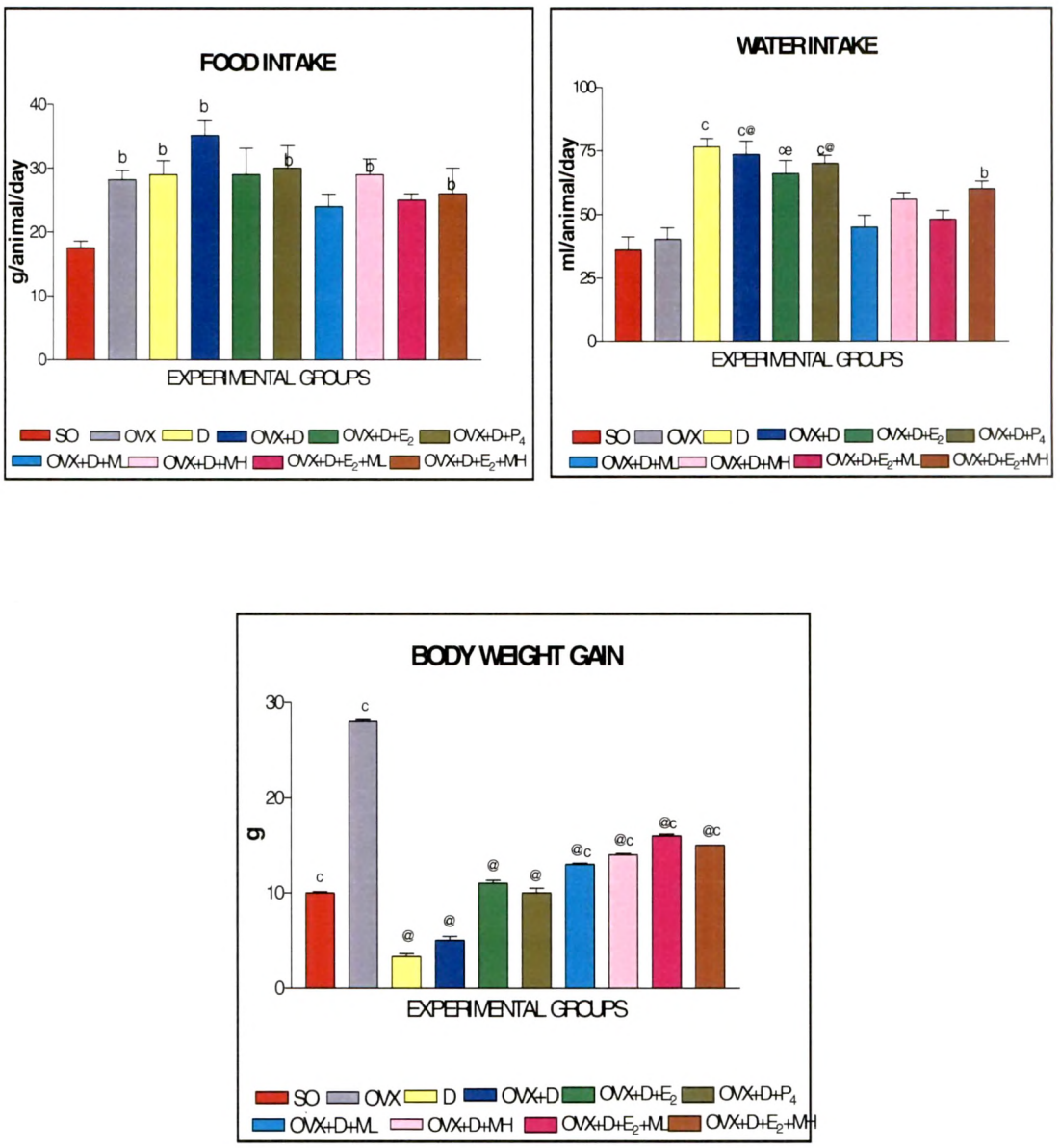
Data are expressed as Mean±SE
$^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{c}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

Table 2: Relative organ weights (g/100g body weight) in all the experimental groups.

GROUPS	Liver	Muscle	Kidney	Uterus
SO	2.25±0.51	0.49±0.002	1.71±0.44	0.16 ± 0.02^{c}
OVX	2.54±0.64	0.52±0.001	1.81±0.36	0.02 ± 0.002^{c}
D	3.35±0.54	1.08 ± 0.004^{c}	$0.89\pm0.0011^{c@}$	0.19±0.023
OVX+D	3.87 ± 0.23^{be}	$1.15\pm0.022^{c@}$	$0.85\pm0.0012^{c@}$	0.01 ± 0.0001^{c}
OVX+D+E_2	$3.12\pm0.04^{a*}$	$1.02\pm0.002^{c@}$	$0.87\pm0.23^{c@}$	$0.42\pm0.01^{c@}$
OVX+ D+P_4	3.56 ± 0.14^{be}	$1.03\pm0.0033^{c@}$	$0.85\pm0.26^{c@}$	$0.32\pm0.02^{c@}$
OVX+ D+ML	3.98 ± 0.23^{be}	$0.87\pm0.0013^{c@}$	$1.10\pm0.16^{c@}$	0.05 ± 0.001^{c}
OVX+ D+MH	3.02±0.44	$1.05\pm0.0031^{c@}$	0.95±0.57	0.065 ± 0.001^{b}
OVX+ D+E_2+ML	2.88±0.35	$0.95\pm0.001^{c@}$	$1.02\pm0.003^{c@}$	$0.42\pm0.03^{c@}$
OVX+ D+E_2+MH	3.22 ± 0.11^{be}	$0.85\pm0.0014^{c@}$	$0.98\pm0.015^{c@}$	$0.38\pm0.01^{c@}$

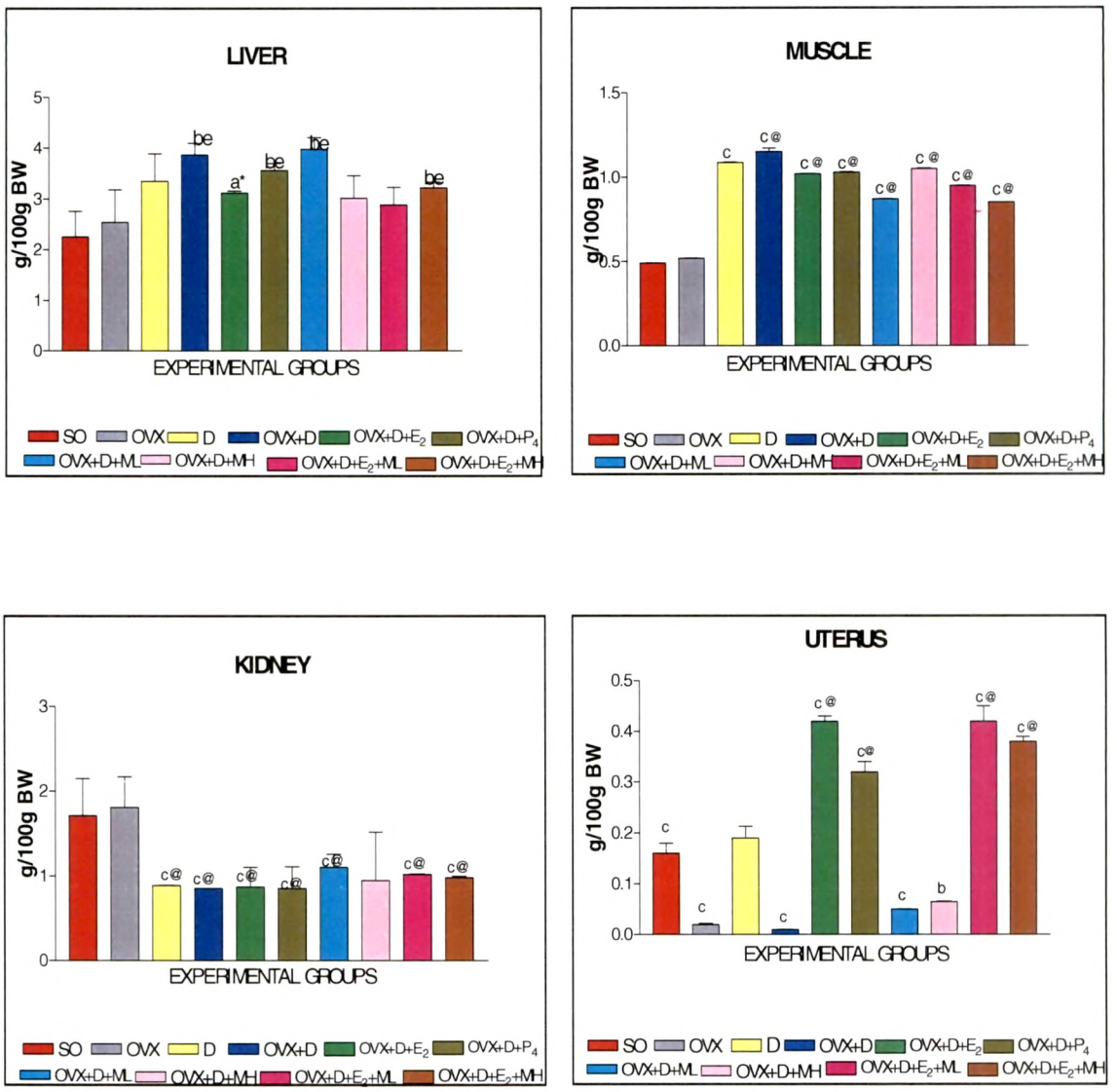
Data are expressed as Mean±SE
$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{c}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

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 [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure 2: Relative organ weights (g/100g body weight) in all the experimental groups.



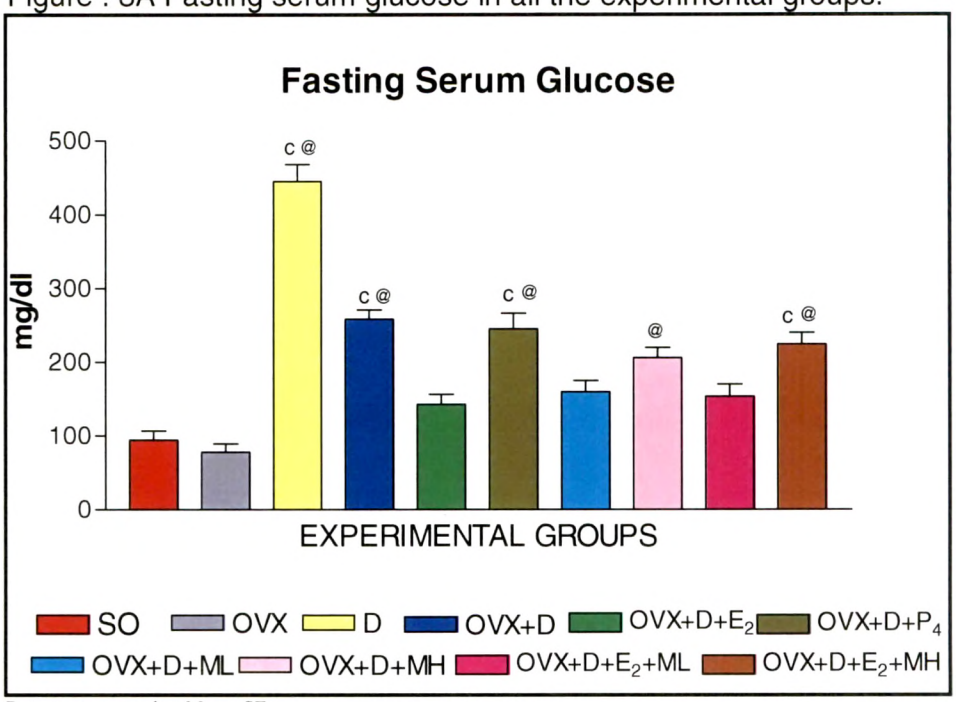
Data are expressed as Mean±SE [a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals

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

GROUPS	Fasting Serum Glucose mg/dl	INSULIN µg/l	FIRI Index	ESTROGEN pg/ml	PROGESTERONE ng/ml
SO	94.33±12.67	0.30±0.02	1.26	24.00±2.22	66.00±6.3
OVX	78.17±11.12	0.50±0.03^{c}	2.16	1.12±0.04^{c}	13.90±1.66^{c}
D	445.00±23.24$^{c@}$	0.16±0.004$^{c@}$	3.39	32.00±1.55	54.68±2.56
OVX+D	258.67±12.56$^{c@}$	0.10±0.012$^{c@}$	1.99	2.02±0.23^{c}	4.32±0.35^{c}
OVX+D+E_2	142.80±13.55	0.30±0.54$^{@}$	2.25	75.00±4.35$^{c@}$	20.70±1.11^{c}
OVX+ D+P_4	245.16±21.21$^{c@}$	0.10±0.02$^{c@}$	1.92	5.55±0.32^{a}	42.80±2.44$^{c@}$
OVX+ D+ML	159.67±15.54	0.16±0.02$^{c@}$	1.42	7.00±0.11^{a}	23.87±3.34$^{@}$
OVX+ D+MH	206.17±13.77$^{@}$	0.13±0.02$^{c@}$	1.49	4.00±0.24^{b}	36.27±0.31^{ce}
OVX+ D+E_2+ML	153.50±16.58	0.20±0.01$^{c@}$	1.70	78.00±3.54$^{c@}$	24.29±8.55^{c}
OVX+ D+E_2+MH	224.50±15.56$^{c@}$	0.16±0.01$^{c@}$	1.98	81.00±4.33$^{c@}$	13.19±2.48^{c}

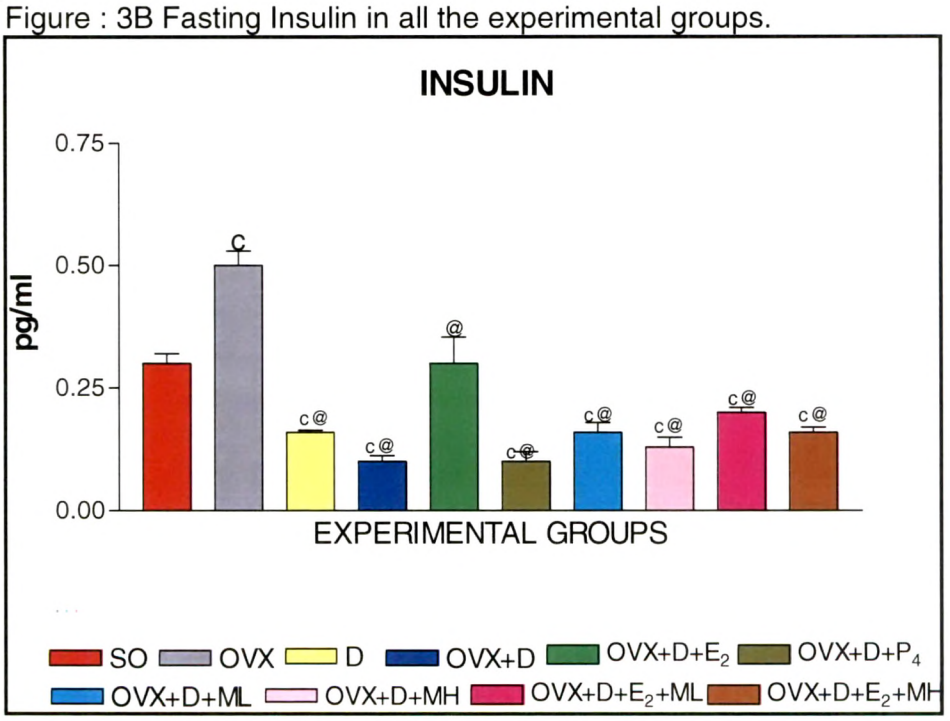
Data are expressed as Mean±SE
$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{e}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

Figure : 3A Fasting serum glucose in all the experimental groups.



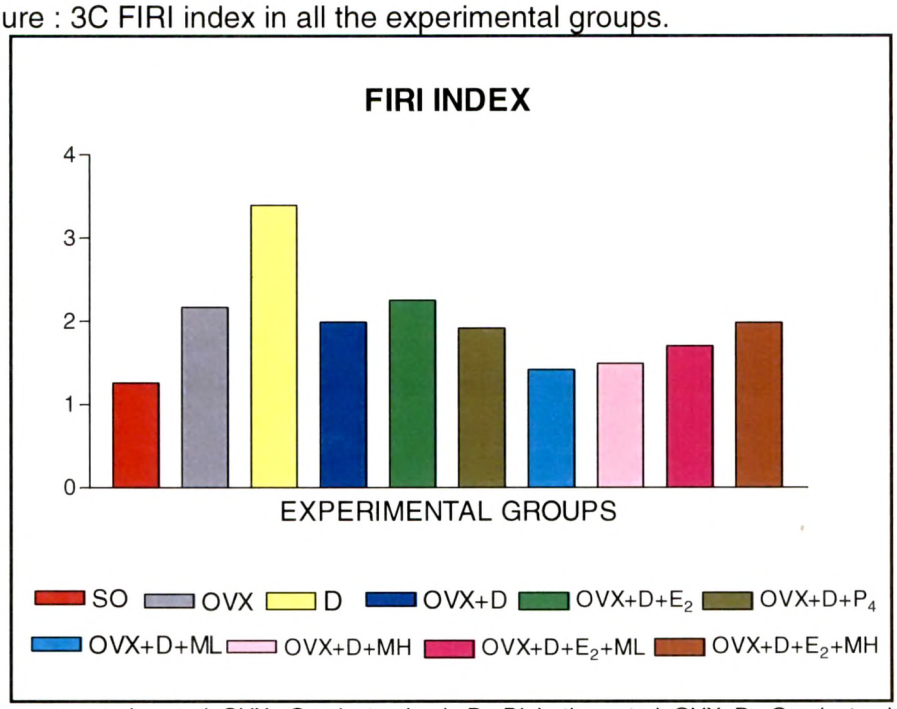
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure : 3B Fasting Insulin in all the experimental groups.



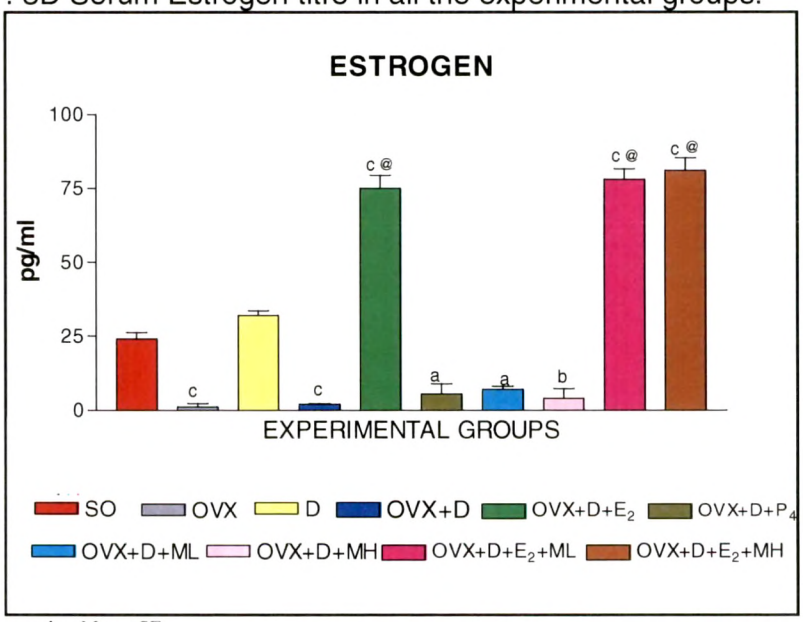
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure : 3C FIRI index in all the experimental groups.



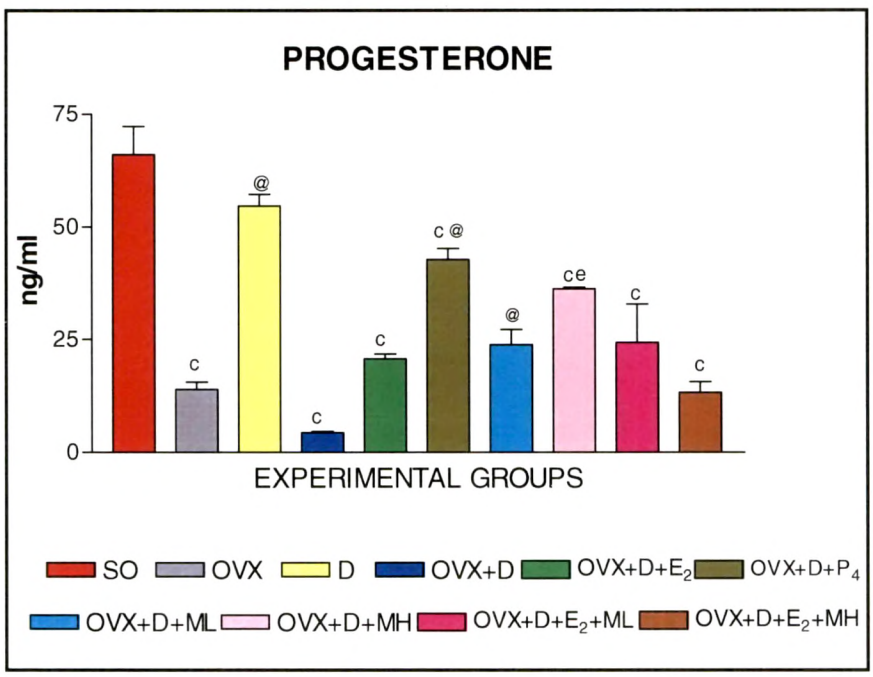
SO= Sham operated control, OVX= Ovariectomized , D= Diabetic control, OVX+D= Ovariectomized diabetic control, OVX+D+E_2= Ovariectomized +Diabetic+ Estrogen, OVX+D+P_4= Ovariectomized Diabetic+ +Progesterone, OVX+D+ML= Ovariectomized+ Diabetic+ Melatonin(Low dose), OVX+ D+MH= Ovariectomized+ Diabetic+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

Figure : 3D Serum Estrogen titre in all the 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<0.001 when compared to ovariectomized animals.

Figure : 3E Serum Estrogen titre in all the 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 [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

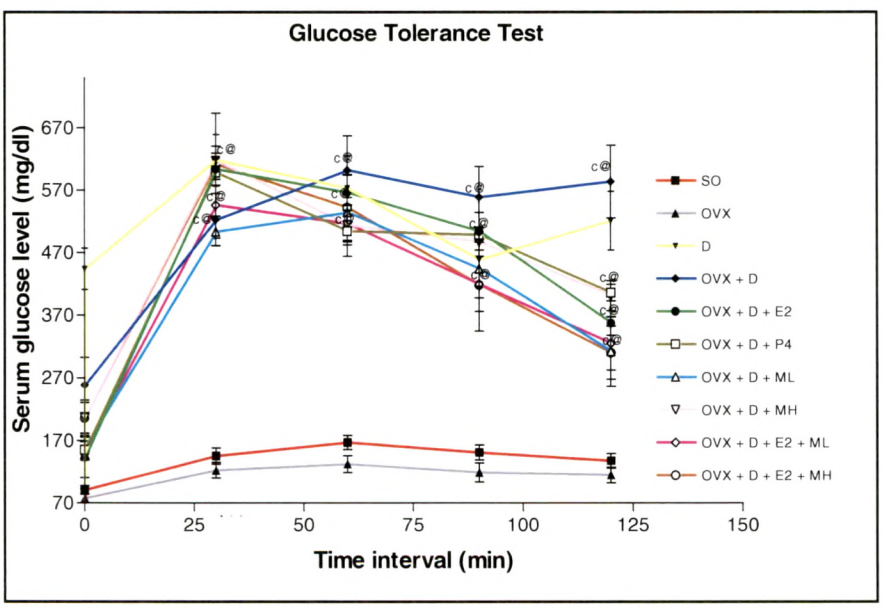
TABLE 4: Glucose tolerance curves of control and experimental rats.

ORAL GLUCOSE TOLERANCE TEST					
GROUPS	0MIN	30MIN	60MIN	90MIN	120MIN
SO	91.00 ± 6.699	145.33 ± 12.890	167.00 ± 11.156	151 .00 ±12.11	137.67 ± 11.858
OVX	77.83 ±15.21	122.50 ±12.33	132.50 ± 13.659	119 .00 ±15.064	115.00 ±12.43
D	444.00 ±33.011$^{c@}$	618 .00 ±41.350$^{c@}$	573.00 ± 52.115$^{c@}$	459.00 ± 42.927$^{c@}$	521 .00 ±47.186$^{c@}$
OVX+D	258.67 ±44.047$^{c@}$	522.67 ± 42.00$^{c@}$	602.00 ± 55.307$^{c@}$	558.67 ± 49.058$^{c@}$	583.83 ± 58.404$^{c@}$
OVX+D+E_2	142.83 ± 32.230	603.67 ± 25.546$^{c@}$	566.16 ± 28.778$^{c@}$	504 .00 ±30.116$^{c@}$	357.50 ± 19.889$^{c@}$
OVX+ D+P_4	155.15 ± 14.107	597.65 ± 10.41$^{c@}$	504.22 ± 17.181$^{c@}$	498.67 ±12.084$^{c@}$	406.11 ± 13.337$^{c@}$
OVX+ D+ML	159.67 ± 21.420	503.50 ± 22.011$^{c@}$	534.67 ± 23.737$^{c@}$	444 .00 ±46.305$^{c@}$	312.23 ±55.659$^{c@}$
OVX+ D+MH	206.16 ± 24.369$^{c@}$	617.00 ±23.529$^{c@}$	514 .00 ± 31.570$^{c@}$	487.00 ± 22.443$^{c@}$	401.00 ± 24.056$^{c@}$
OVX+ D+E_2+ML	155.15 ± 14.307	546.00 ± 52.711$^{c@}$	516.00 ± 52.05$^{c@}$	419.36 ± 43.429$^{c@}$	324.83 ± 42.424$^{c@}$
OVX+ D+E_2+MH	205.83 ± 28.307$^{c@}$	611.00 ± 82.711$^{c@}$	542.00 ±52.052$^{c@}$	418.00 ±73.429$^{c@}$	310 .00 ±42.424$^{c@}$

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.

Fig 4: Glucose tolerance curves of control and experimental rats.



Data are expressed as Mean±SE

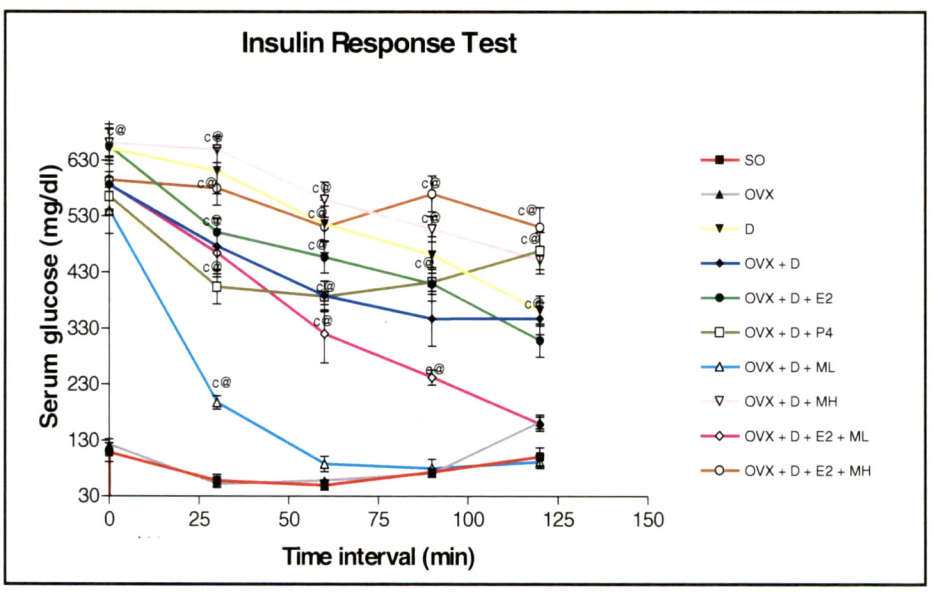
^{c}p<0.001 when compared to sham operated control and $^{@}$p<0.001 when compared to ovariectomized animals.

Table 5: Insulin response curves in control and experimental rats.

INSULIN RESPONSE TEST					
GROUPS	0MIN	30MIN	60MIN	90MIN	120MIN
SO	108.00 ± 16.6	50.00 ± 10.8	50.00 ± 9.156	73 .00 ±5.111	100.67 ± 15.858
OVX	121.67 ± 10.210	52.33 ± 6.336	58.16 ± 3.659	71.50 ±5.064	162.67 ± 12.432
D	652 .00 ± 43.011 $^{c@}$	611.00 ±41.350 $^{c@}$	516.00 ±32.115 $^{c@}$	461.00 ± 32.927 $^{c@}$	361.00 ± 27.186 $^{c@}$
OVX+D	585.50 ± 44.04 $^{c@}$	476.00 ±49.148 $^{c@}$	388.00 ± 25.307 $^{c@}$	346.83 ±49.058 $^{c@}$	347.33 ±28.404 $^{c@}$
OVX+D+E_2	655.33 ± 32.23 $^{c@}$	501.00 ±25.546 $^{c@}$	457.00 ±28.778 $^{c@}$	408.83 ± 30.116 $^{c@}$	308 .00 ±29.889 $^{c@}$
OVX+ D+P_4	565.16 ± 34.107 $^{c@}$	403.66 ±30.409 $^{c@}$	386.56 ± 27.181 $^{c@}$	412.66 ±22.084 $^{c@}$	468.33 ± 33.337 $^{c@}$
OVX+ D+ML	539.83 ± 41.402 $^{c@}$	197.33 ± 12.011	87.67 ±13.737	80.00 ±16.305	91.33 ± 11.659
OVX+ D+MH	661.50 ± 24.369 $^{c@}$	649.33 ± 23.529 $^{c@}$	559.67 ± 31.570 $^{c@}$	507.00 ± 22.444 $^{c@}$	451.33 ± 24.056 $^{c@}$
OVX+ D+E_2+ML	586.83 ± 48.307 $^{c@}$	463.83± 42.711 $^{c@}$	320.00 ±52.052 $^{c@}$	242.00 ± 13.429 $^{c@}$	158.83 ± 12.424
OVX+ D+E_2+MH	594.67 ± 34.106 $^{c@}$	579.95 ± 30.409 $^{c@}$	511.50 ± 47.181 $^{c@}$	570.00 ±32.084 $^{c@}$	510.33 ± 35.337 $^{c@}$

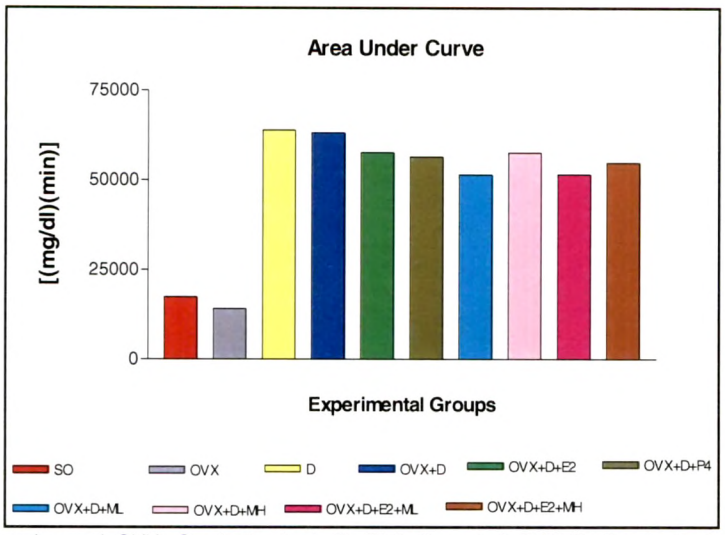
Data are expressed as Mean±SE
, ^{c}p<0.001 when compared to sham operated control and $^{@}$p<0.001 when compared to ovariectomized animals.

Fig 5: Insulin response curves in control and experimental rats.



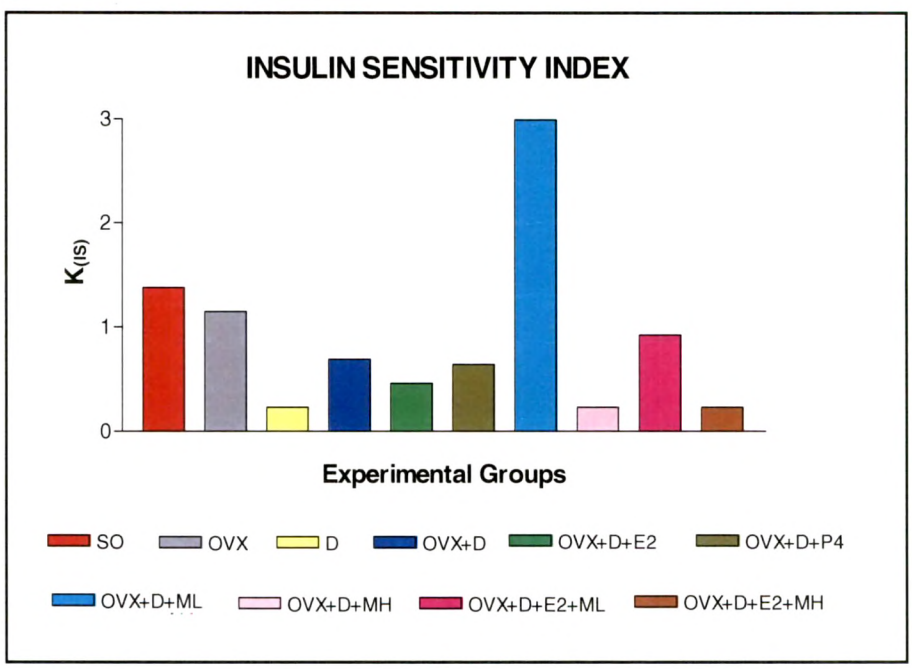
Data are expressed as Mean±SE
^{c}p<0.001 when compared to sham operated control and $^{@}$p<0.001 when compared to ovariectomized animals.

Fig 4A: Area under curve for Glucose tolerance test in control and treated rats.



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

Fig 5A: Insulin sensitivity index in control and experimental rats.



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

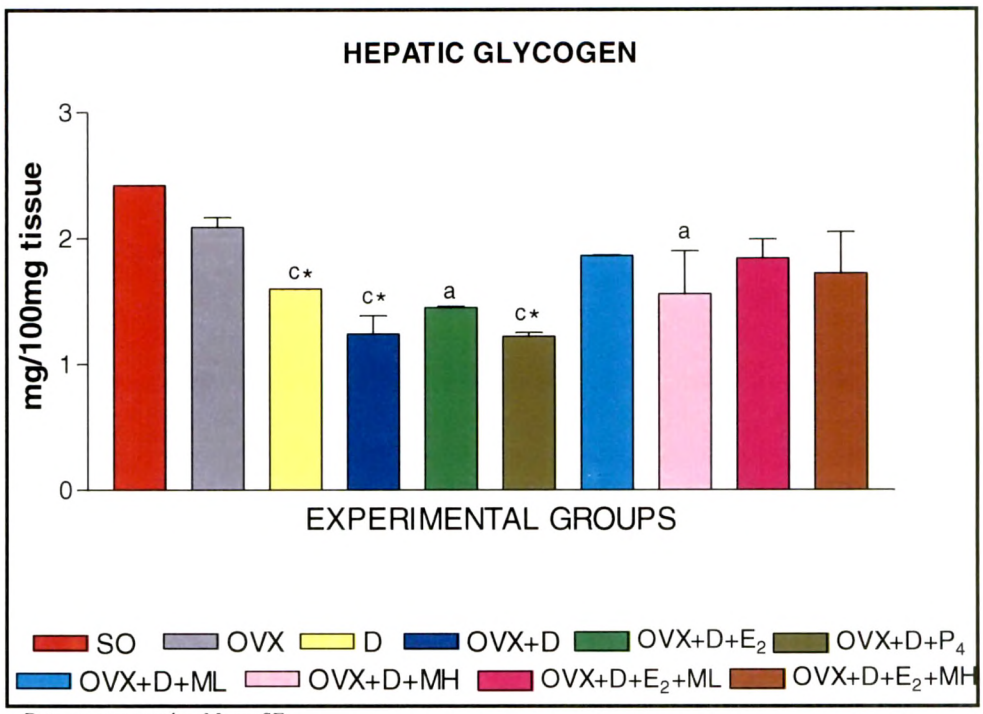
Table 6: Hepatic Glycogen content and glycogen phosphorylase and G-6-Pase activity in control and experimental groups.

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±0.002	0.128 ±0.001	0.23 ±0.0182
OVX	2.09±0.08	0.149 ± 0.003^{c}	0.28 ±0.0220
D	$1.60\pm0.002^{c*}$	$0.190\pm0.001^{c@}$	0.32 ± 0.012^{b}
OVX+D	$1.24\pm0.15^{c*}$	$0.212\pm0.003^{c@}$	0.38 ± 0.012^{b}
OVX+D+E_2	1.45 ± 0.01^{a}	$0.188\pm0.0023^{c@}$	0.32±0.0031
OVX+ D+P_4	$1.22\pm0.035^{c*}$	$0.198\pm0.0021^{c@}$	0.31±0.003
OVX+ D+ML	1.86±0.005	0.133 ± 0.0033^{e}	0.25±0.0261
OVX+ D+MH	1.56 ± 0.34^{a}	0.157 ± 0.0002^{c}	0.30±0.056
OVX+ D+E_2+ML	1.84±0.15	$0.168\pm0.002^{c@}$	0.25±0.0011
OVX+ D+E_2+MH	1.72±0.33	$0.176\pm0.0033^{c@}$	0.35 ± 0.0002^{a}

Data are expressed as Mean±SE

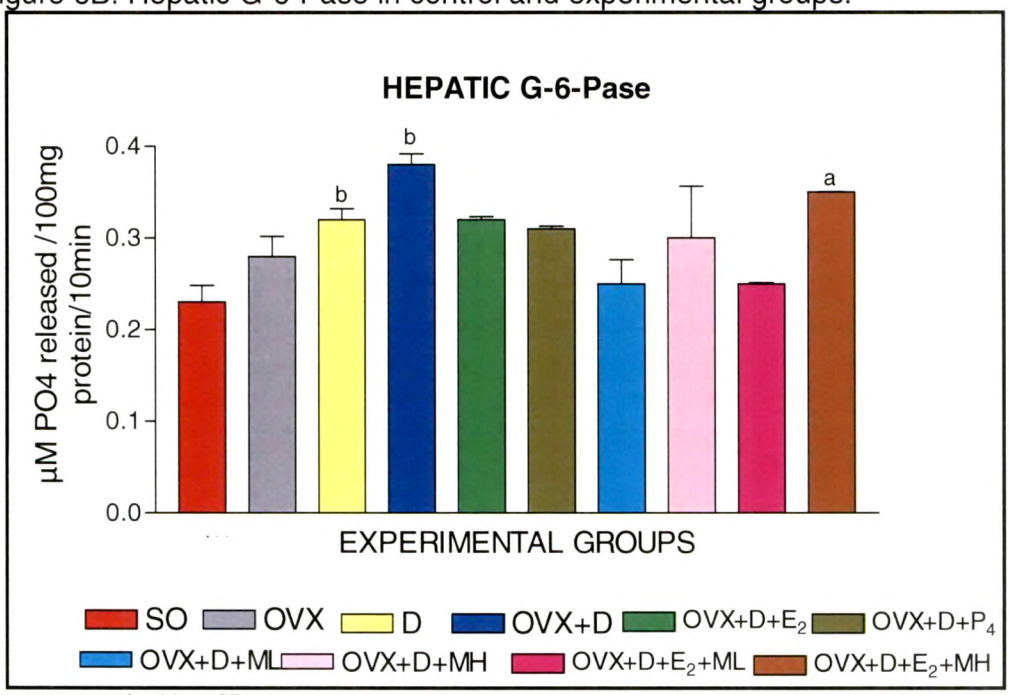
$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{e}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

Figure 6A: Hepatic Glycogen content in control and experimental groups.



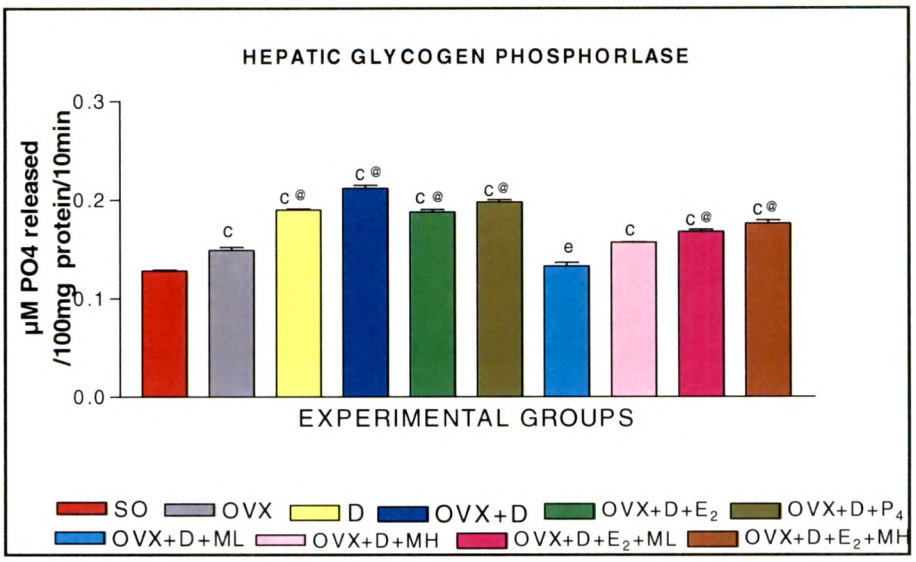
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

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



Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01when compared to sham operated control

Figure 6C: Hepatic Glycogen Phosphorylase in control and experimental groups



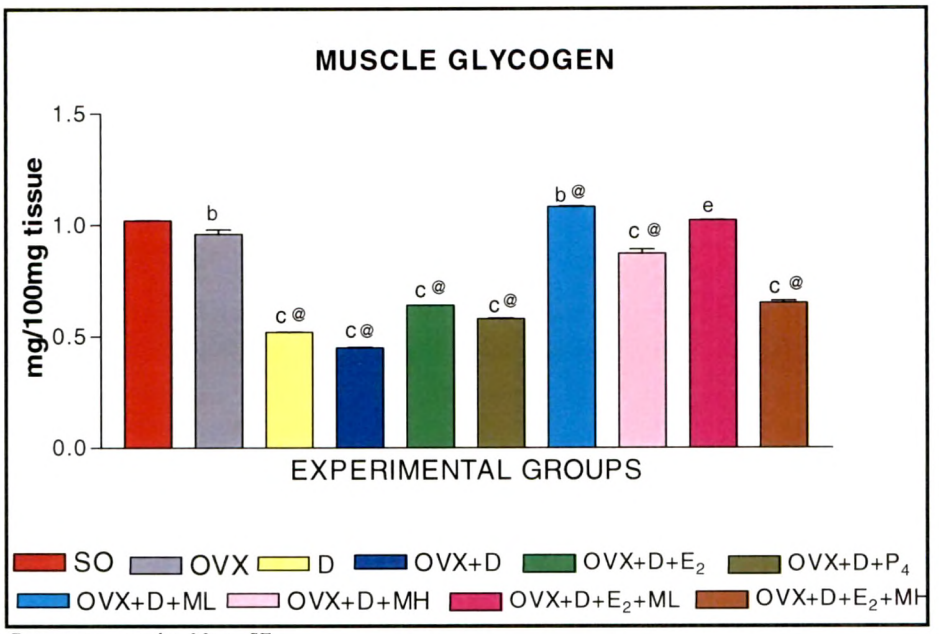
Data are expressed as Mean±SE
[c]p<0.001 when compared to sham operated control and [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

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±0.002	0.25 ±0.001
OVX	0.96±0.02[b]	0.28 ±0.003[a]
D	0.52±0.001[c@]	0.35±0.0021[c@]
OVX+D	0.45±0.002[c@]	0.35±0.003[c@]
OVX+D+E_2	0.64±0.001[c@]	0.32±0.002[c@]
OVX+ D+P_4	0.58±0.0013[c@]	0.30±0.001[c]
OVX+ D+ML	1.08±0.0025[b@]	0.23±0.01[@]
OVX+ D+MH	0.87±0.021[c@]	0.29±0.001[c]
OVX+ D+E_2+ML	1.02±0.001[e]	0.28±0.012[a]
OVX+ D+E_2+MH	0.65±0.01[c@]	0.30±0.001[c]

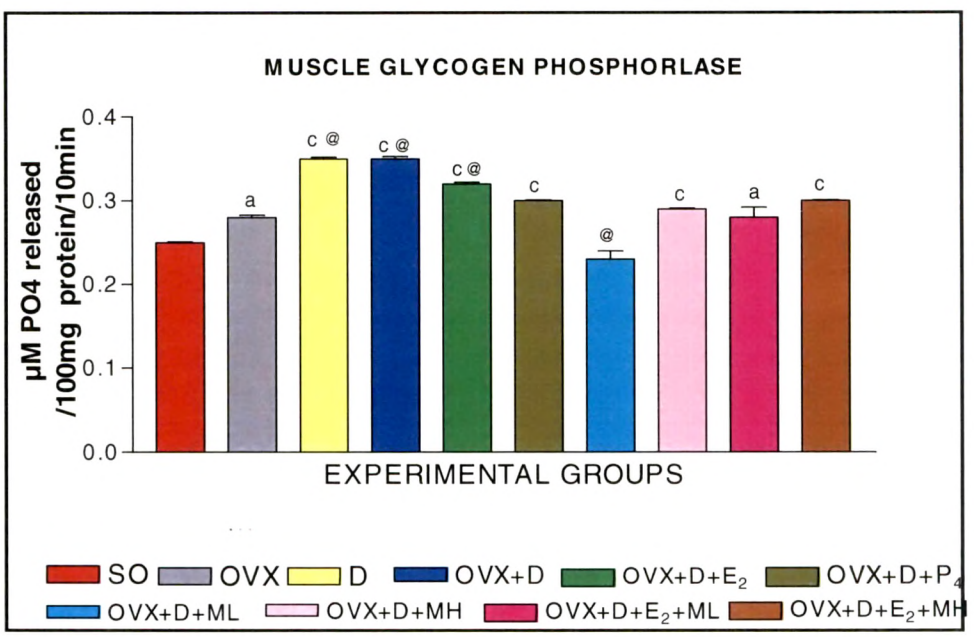
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Fig 7A : Changes in muscle glycogen contents in control and experimental animals



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

Fig 7A : Changes in muscle phosphorylase activity in control and experimental animals



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

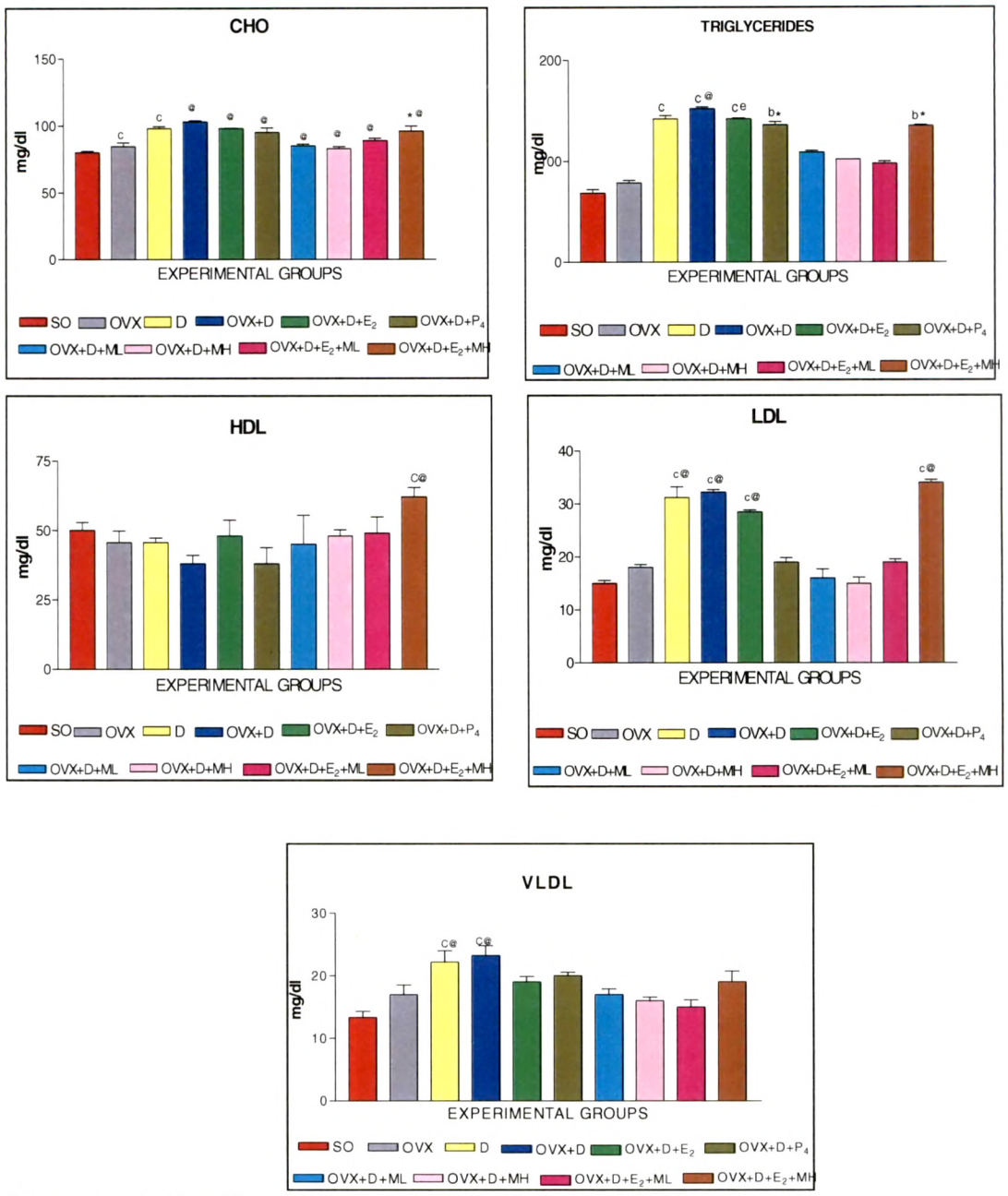
Table 8: Changes in serum lipid profile in control and experimental groups

SERUM LIPID PROFILE: (mg/dl)					
GROUPS	CHO	TG	HDL	LDL	VLDL
SO	80.00 ±1.15	68.67 ±3.74	50.00 ±2.90	15.00±0.57	13.33±0.97
OVX	84.67 ±2.73^{c}	78.67 ±2.58	45.67 ±4.16	18.00±0.57	17.00±1.52
D	98.00±1.71^{c}	142.00±13.18^{c}	45.66±1.64	31.21±2.03$^{c@}$	22.22±1.76$^{c@}$
OVX+D	103.00±10.88$^{@}$	152.00±11.89$^{c@}$	38.00±3.05	32.21±0.49$^{c@}$	23.24±1.57$^{c@}$
OVX+D+E_2	98.00±0.33$^{@}$	142.00±10.98^{ce}	48.00±5.7	28.45±0.39$^{c@}$	19.00±0.88
OVX+ D+P_4	95.00±3.51$^{@}$	136.00±3.18^{b*}	38.00±5.78	19.00±0.88	20.00±0.57
OVX+ D+ML	85.00±1.45$^{@}$	109.00±5.59	45.00±4.42	16.00±1.73	17.00±0.88
OVX+ D+MH	83.00±1.45$^{@}$	102.00±8.30	48.00±2.31	15.00±1.15	16.00±0.57
OVX+ D+E_2+ML	89.00±1.73$^{@}$	98.00±2.02	49.00±5.78	19.00±0.57	15.00±1.15
OVX+ D+E_2+MH	96.00±3.79$^{*@}$	135.00±5.99^{b*}	62.00±3.46$^{c@}$	34.00±0.57$^{c@}$	19.00±1.73

Data are expressed as Mean±SE

$^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{c}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

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



Data are expressed as Mean±SE

$^{c}p<0.001$ when compared to sham operated control and $^{*}p<0.05$, $^{c}p<0.01$, $^{@}p<0.001$ when compared to ovariectomized animals.

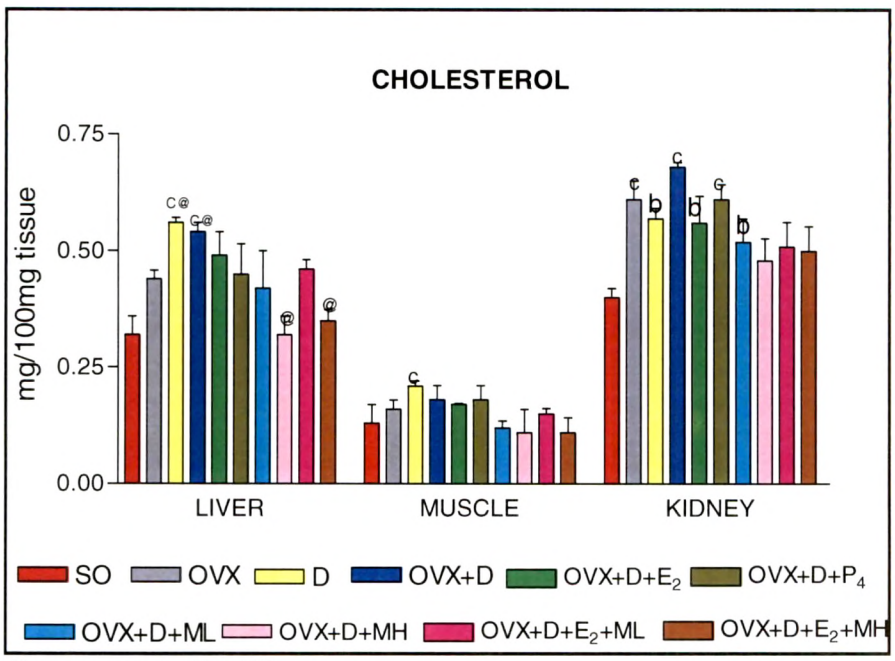
Table 9: Tissue lipid and cholesterol contents in control and experimental groups

	Cholesterol (mg/100mg tissue)			LIPID(mg/100mg tissue)		
GROUPS	LIVER	MUSCLE	KIDNEY	LIVER	MUSCLE	KIDNEY
SO	0.32 ±0.04	0.13 ±0.04	0.40 ±0.02	4.01±0.071	1.86±0.043	0.85±0.06
OVX	0.44 ±0.18 $^{c@}$	0.16 ±0.02	0.61 ±0.04 ^{c}	4.85±0.054 ^{c}	1.95±0.045	0.88±0.054
D	0.56±0.011 $^{c@}$	0.21±0.011 ^{c}	0.57±0.22 ^{b}	6.23±0.021 $^{c@}$	2.02±0.03	0.93±0.045
OVX+D	0.54± 0.02	0.18±0.031	0.68±0.01 ^{c}	5.23±0.05 ^{c}	2.22±0.04	0.97±0.021
OVX+D+E_2	0.49±0.05	0.17±0.002	0.56±0.058 ^{b}	4.88±0.056 ^{c}	1.99±0.055	0.81±0.057
OVX+ D+P_4	0.45±0.065	0.18±0.031	0.61±0.033 ^{c}	5.12±0.068 ^{c}	2.11±0.065	0.89±0.061 ^{a}
OVX+ D+ML	0.42±0.08	0.12±0.015	0.52±0.05	4.23±0.065	1.89±0.023	0.77±0.047
OVX+ D+MH	0.32±0.04 $^{@}$	0.11±0.05	0.48±0.047	4.11±0.057	1.75±0.061	0.79±0.062
OVX+ D+E_2+ML	0.46±0.021	0.15±0.012	0.51±0.052	4.35±0.085	1.66±0.078	0.88±0.088
OVX+ D+E_2+MH	0.35±0.024 $^{@}$	0.11±0.032	0.50±0.053	4.67±0.098	1.68±0.044	0.79±0.021

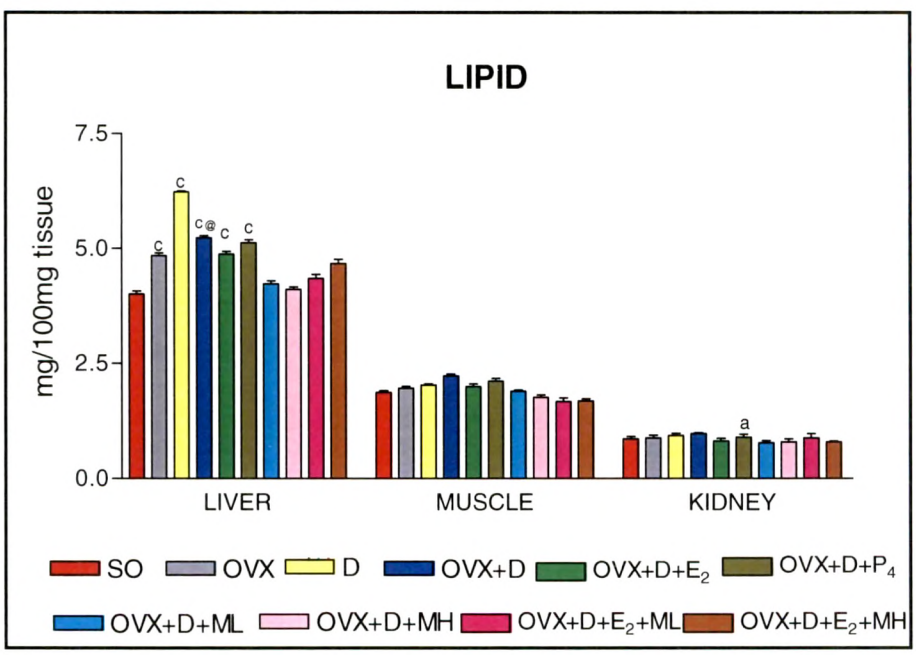
Data are expressed as Mean±SE

$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ when compared to sham operated control and $^{@}p<0.001$ when compared to ovariectomized animals.

Figure 9: Tissue lipid and cholesterol contents 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<0.001 when compared to ovariectomized animals.



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.

Discussion

In a previous study, we had shown MST to be more potent than ERT and even ERT+MST in ameliorating OVX induced glycaemic dysregulation and alterations in carbohydrate metabolism and tissue and serum lipid profile, suggesting MST as an effective alternative in combating post menopausal metabolic disturbances. The present study further shows that OVX induced changes get compounded by diabetic induction in such individuals which can also be effectively counteracted by MST or a combination of ERT+MST rather than ERT. Food and water intake and liver and muscle mass which are all increased in OVX and D animals show an additive effect in ovariectomized diabetic animals. In contrast to the liver and muscle weights, renal weight increases due to OVX but decreases in diabetes and the OVX diabetic animals had a renal mass similar to that of D animals. All these changes with reference to food and water intake and organ weights in OVX diabetic animals are more effectively reversed in MST_L and ERT+MST_L animals in that order. Even MST_H and ERT+MST_H are noted to be more effective than ERT alone.

Very little information is available on the use of ERT in diabetic post-menopausal women (PMW) implying a compelling need to evaluate the efficacy of treatment schedules in diabetic PMW. From the available reports it can be surmised that ERT at low doses may have some beneficial effects in PMW with metabolic syndrome but conventional ERT is often beset with adverse effects on glucose clearance, triglycerides and C-reactive protein content (L'Hermite *et al.*, 2008). This negative dampner finds support in the reported worsening of insulin resistance and adipocytokine parameters with

oral estradiol therapy (Choi *et al.*, 2005). Our earlier study had explored the dose dependent potency of melatonin as an alternative to ERT in alleviating altered insulin sensitivity, glucoregulation and dyslipidemia in ovariectomized rats (Chapter 1). As a sequel to these findings and in view of the available literature based on ours and others works supporting the ameliorative potentials of melatonin in diabetic alterations (Montilla *et al.*, 1998; Sudnikovich *et al.*, 2007; Singh *et al.*, 2010 a, b, c). Whereas both ovariectomized non-diabetic and diabetic rats have insignificant levels of E_2 and P_4, ovary intact diabetic animals tended to record an increase in E_2 level and decrease in P_4 level. Estrogen and progesterone supplemented rats had higher levels of three hormones in the respective groups as has also been shown by Choi *et al.* (2005), Ordonez, (2006). Ovariectomized animals tend to show a lowered serum glucose level which can be related with the increased insulin level in these animals. The increase in serum insulin titre and increased FIRI index seen herein find corroboration from the reported whole body insulin resistance and elevated serum insulin level in rats 5 weeks post ovariectomy (Lui *et al.*, 2004). Ovariectomized diabetic animals show a lower fasting hyperglycaemia compared to ovary intact diabetic animals which is not seen in the fed state. Obviously, a fasted state seems to have favourable effect on glycaemic status in the absence of ovarian sex hormones. This tends to suggest some possible difference in starvation physiology with reference to glucose metabolism in presence or absence of ovarian sex hormones. A cursory glance at serum glucose and insulin levels and parameters of carbohydrate metabolism in OVX diabetic animals subjected to

various therapies suggests MST_L to be one of the most meaningful followed by MST_L+ERT, MST_H and MST_H+ERT in that order. Apparently, melatonin alone or combination with E_2 seems to be the therapy of choice for alleviating diabetic PM symptoms of hyperglycaemia and alterations in carbohydrate metabolism. The favourable responses are clearly indicated by significant anti-hyperglycaemic effects and increased hepatic and muscle glycogen contents with decreased glycogen phosphorylase activity and hepatic G-6-Pase activity. Though ERT is seen to be effective in mitigating the complications of both E_2 deficiency and diabetic manifestations, is nevertheless less effective compared to MST, MST_L+ERT. Despite the fact that insulin levels are lower in MST and MST+ERT combinations, compared to ERT, both glucose tolerance and insulin response curves are much better than in ERT. The maximal sensitivity and the minimal resistance indices towards insulin shown by melatonin supplementation either alone or in combination with E_2 provides supportive evidence for the mitigative effects on glycaemic dysregulation and disturbances in carbohydrate metabolism characteristic of estrogen deficient diabetic animals. No doubt, ERT has also favourable influences as seen herein and, as such, may decrease insulin resistance in diabetic and OVX rats by increasing β cell proliferation though induction of IRS-2 and pancreatic homeodomain protein I via activation of cAMP dependent binding proteins (Choi *et al.*, 2005; Ordóñez *et al.*, 2007).

Classical therapy for post-menopausal symptoms and POF/surgical menopause is ERT/HRT and, many studies have shown favourable influences in the form of promoting whole body glucose metabolism decreasing insulin

resistance, increasing insulin secretion, preventing diabetic induction etc. The findings from all of these studies are also reported with riders cautioning the use of ERT/HRT in terms of dosage, duration, timing of initiation, individual disposition etc as otherwise the above favourable influences may be negatively impacted jeopardizing the health and safety of post-menopausal and POF/ surgical menopause individuals (Van *et al.*, 2003; Margolis *et al.*, 2004; Godsland, 2005; Goswami and Conway, 2005, 2007; Sanchez-Mateos *et al.*, 2007; Wedisinghe and Perera, 2009; Bhattacharya and Jha, 2010). Apparently many of them have advocated the need to explore alternate therapies and in fact, one of the studies did even suggest the possible safety and utility of a combination of E_2 and melatonin to reap the benefits of both (Goswami and Conway, 2007). Our findings clearly favour MST_L alone in ameliorating all diabetic manifestations affecting glucoregulation and carbohydrate metabolism in the surgical menopause and, in POF individuals not committed to fertility improvement.

The present results on serum lipid profile and tissue cholesterol and TG load, indicate that both OVX and D have an elevating effect with OVX diabetic animals registering the maximum increment. Such an increment in lipid profile can not only lead to increasing insulin resistance and exaggerated type 2 diabetic complications but also pre-dispose PMW and POF/surgical menopause individuals to cardio-vascular disorders. Management of elevation in serum lipid profile and increasing adiposity is integral in maintaining normal health of PMW and subjects suffering from POF/surgical menopause. In both ovariectomized rats as well as diabetic PMW and POF/ surgical menopause

individuals, ERT/HRT has been routinely attempted but with unsatisfactory end points and alternate modes of therapy has been suggested in most of these studies (Lundeen *et al.*, 1997; Manwaring *et al.*, 2000; Crespo *et al.*, 2002; Tuna *et al.*, 2010). In this context our present study, MST with both low and high doses demonstrate excellent lipid and cholesterol lowering effects in OVX diabetic rats suggesting the possible application of MST in countering the lipid and cholesterol elevating effects of ovariectomy and diabetes. Even the combination therapies with MST and E_2 are significantly better than ERT alone. Apparently, MST or a combinational therapy involving melatonin and E_2 seems to be a potent mode to lower lipid and cholesterol profile in PMW as well as in POF/surgical menopause females depending on the situation or individual choice.

Association of type I diabetes with POF has now been clearly established as a relation between POF and changes in HLA regions of chromosome 6 which confer susceptibility to type I diabetes has been observed (2). Further, ineffectiveness of E_2 to protect against complications of diabetes as well as of diabetes alongwith E_2 deficiency as in PMW or POF/ surgical menopause females or even in OVX diabetic rats has been adequately documented. These studies have highlighted cases such as diabetes abolishing the vascular effects of estrogen in female rats (Bolego *et al.*, 1999), E_2 mitigating only some of the health risks such as premature death, cardiovascular disorders, neurologic complications, osteoporosis, psycho-sexual dysfunctions and mood disorders usually found associated with premature menopause or early menopause whether spontaneous or

induced (Shuster *et al.*, 2010), inability of E_2 to prevent decrease in bone strength, though able to maintain bone mineral density, in OVX diabetic rats (Verhaeghe *et al.*, 1997) and failure of E_2 to effect neuroprotection in OVX diabetic rats (Santizo *et al.*, 2002; Yong *et al.*, 2005). All these observations prelude the long term use of ERTs as an option in both spontaneous as well as induced menopause and suggest the explicit need to explore efficient and effective alternative therapies to combat various deleterious and discomforting consequences of natural or premature menopause due to POF or surgical intervention further compounded by diabetic insult. Melatonin supplementation seems to be an ideal and safe alternative in PMW or POF women suffering from diabetes not only because of the herein recorded favourable effects on insulin sensitivity, glucoregulation, carbohydrate and lipid metabolisms but also due to the many reports of heightened melatonin's versatility in preventing/ameliorating cardio-vascular disorders, osteoporosis and neurological lesions. (Montilla *et al.*, 1998; Goswami and Conway, 2007; Sudnikovich *et al.*, 2007). In cases of POF, either MST, alone or a combination MST and E_2 can be advocated depending on the need of the individual in terms of fertility management. For those not in need of fertility management willfully (follicular POF) or by design (a follicular POF), MST_L is the therapy of choice while those requiring fertility maintenance (follicular POF), a combination of MST with simultaneous low dose estrogen replacement may be ideal in view of the ability of melatonin to nullify the harmful effects of estrogen.

Overall, the present study provides strong evidence for melatonin supplementation therapy alone or in combination with low doses of estrogen (depending on the need) as the most potent alternative to ERT for post-menopausal diabetic women or women suffering from premature ovarian failure or surgical menopause in combating the symptoms and consequences of E_2 deficiency compounded by diabetes.