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Melatonin supplementation therapy as a potent alternative to ERT in ovariectomized rats

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ABSTRACT

Aim To evaluate the efficacy of melatonin supplementation therapy as an alternative to estrogen replacement therapy in an ovariectomized rat model and to assess diabetogenic metabolic dysregulation caused by estrogen deficiency in postmenopausal individuals.

Methods Ovariectomized adult Wistar rats were treated with either estrogen/progesterone, melatonin or a combination of estrogen and melatonin. Body weight gain, feed efficiency, serum glucose, insulin, glucose tolerance and insulin response, serum and tissue lipids, tissue glycogen contents and activities of glycogen phosphorylase and glucose-6-phosphatase were analyzed in all the experimental groups.

Results Ovariectomized animals showed increased body weight gain, feed efficiency, fasting insulin resistance, greater area under curve for the glucose tolerance test, higher serum and tissue lipids and reduced glycogen content and insulin sensitivity. A low dose of melatonin was more efficient than estrogen in reversing all the ovariectomy-induced changes. The combination of estrogen + melatonin was found to be best in correcting glycemic dysregulation while high doses of melatonin could effectively regulate dyslipidemia.

Conclusion The present study provides strong evidence for melatonin supplementation therapy to be more potent and effective in comparison to estrogen replacement therapy due to its single-handed ability to revert all the ovariectomy-induced changes. No reported side-effect or long-term effect of melatonin, against the known effects of estrogen replacement therapy, make it more attractive as a candidate to treat postmenopausal symptoms.

INTRODUCTION

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, mainly attributable to the decreasing levels of sex steroids as a prelude to the onset of menopause. Menopause as a midlife event ushers in a series of physiological changes that make the individual susceptible to various diseases in the aftermath of altered internal milieu. Despite reduced pancreatic insulin secretion, there is little change in the overall circulating insulin level due to poor elimination of secreted insulin[1]. However, with increase in age, progressive insulin resistance develops that predisposes postmenopausal women towards development of type II diabetes. An overall reduction in insulin-mediated whole body glucose uptake associated with loss of ovarian function[2] makes the condition even worse in women with type I diabetes that can further hamper glycemic regulation and reduce insulin sensitivity[3]. 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*[1]. There is a reported eight-fold increase in the incidence of endometrial cancer in women who are on prolonged unopposed estrogen therapy[4,5]. The benefit of estrogen used in HRT is controversial, with reports

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indicating both beneficial (lower doses) and detrimental (higher doses) effects on insulin sensitivity[6,7]. Although a combination of dihydroprogesterone and estradiol appears capable of reversing menopause-associated changes in insulin secretion and elimination[8,9], medroxyprogesterone acetate reportedly causes deterioration of insulin resistance[10,11]. Apart from the by now established cancer risk, the above reports of disturbances in metabolism warrant the need for greater caution while attempting HRT. To this end, there is need to explore possible alternatives to HRT/estrogen replacement therapy (ERT) for alleviation of postmenopausal 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. Night-shift workers have shown higher levels of insulin, glucose and triacylglycerol after a night-time meal than after a daytime meal, suggestive of desynchronization of bodily functions, which have been related to a higher incidence of heart disease and metabolic disturbances such as diabetes[12]. The potent role of melatonin in stimulating glucose transport through the IRS I/PI-3-kinase pathway in skeletal muscles suggests its therapeutic value against glucose dyshomeostasis and diabetes[13–15]. Both exposure to light at night and aging are known to lower melatonin levels and contribute to the development of diabetes. The present study in this context is an attempt to evaluate the therapeutic value of melatonin as an alternative to combat symptoms associated with conditions of sex steroid deficiency.

MATERIALS AND METHODS

Experimental animals

Female albino Wistar rats of 200–250 g body weight were used for the study. Animals were maintained under 12 h : 12 h light–dark cycle and 21–23°C temperature regimen throughout in accordance with CPCSEA guidelines, and the animal experiments were approved by the animal ethical committee of the Department of Zoology, The M. S. University of Baroda, Vadodara (Approval no 827/ac/04/CPCSEA). Throughout the experimental period, animals were provided with standard rat chow and water *ad libitum*. The rat chow was purchased from M/S Pranav Agro limited, Baroda.

Ovariectomy

Ovariectomy or sham operation for the experiments was performed as per previous reports[16,17]. Bilateral ovariectomy was performed under anesthetic condition with a single midventral incision. For sham operation, ovaries were located but not removed. Following surgery, animals were kept in a resting phase for about 20 days so as to enable them to recover completely from surgical stress and also to allow the circulating sex steroid levels to diminish.

Experimental design

Animals were divided into eight groups of six animals each:

(1) Group I (SO): Sham-operated control animals injected intraperitoneally (i.p.) with the vehicle (saline) for 15 days;
(2) Group II (OVX): Ovariectomized animals administered with saline for 15 days;
(3) Group III (OVX + E2): Ovariectomized animals subjected to ERT at a dose of 30 μg/kg body weight of 17β-estradiol (i.p.) for a period of 15 days;
(4) Group IV (OVX + P4): Ovariectomized animals subjected to progesterone replacement (PRT) (i.p.) at a dose of 20 mg/kg body weight for a period of 15 days;
(5) Group V (OVX + ML): Ovariectomized animals subjected to low-dose melatonin (ML) supplementation as an alternative at a dose of 1 mg/kg body weight (i.p.) for a period of 15 days;
(6) Group VI (OVX + MH): Ovariectomized animals subjected to high-dose melatonin (MH) supplementation at a dose of 10 mg/kg body weight (i.p.) for a period of 15 days;
(7) Group VII (OVX + E2 + ML): Ovariectomized animals subjected to a combination of ML (1 mg/kg) and ERT (30 μg/kg) for a period of 15 days;
(8) Group VIII (OVX + E2 + MH): Ovariectomized animals subjected to a combination of MH (10 mg/kg) and ERT (30 μg/kg) for a period of 15 days.

Fasting blood glucose was checked at regular intervals. Body weight, food intake and water intake were recorded on a daily basis for the entire duration of treatment schedule.

Oral glucose tolerance test and insulin response test

At the end of the treatment period, animals were subjected to oral glucose tolerance test (OGTT) and insulin response test (IRT) according to the procedure mentioned in our earlier work[14,17,18]. The area under the curve (AUC) for both OGTT and IRT was calculated by using the Graph Pad Prism Version 3.0 for Windows, Graph Pad software, San Diego, CA, USA.

Biochemical estimations

At the end of the experimental period, animals were starved overnight and sacrificed by decapitation. Blood was drawn from the jugular vein and serum was separated for various assays. Liver, muscle, kidney, uterus and adipose tissue were dissected out, washed in saline, blotted dry and weighed. Blood glucose levels were estimated by the glucose oxidase method[19] using kit obtained from Aggape Diagnostics. Insulin was estimated using an ELISA-based assay kit (Rat Insulin ELISA kit from Mercodia, Sweden). Serum lipid profile was checked wherein total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL) and very

low density lipoprotein (VLDL) cholesterols were assayed by using appropriate kits. Glycogen was assayed by the method of Seifter and colleagues[20], glycogen phosphorylase by that of Cahill and colleagues[21] and glucose-6-phosphatase by the method of Harper[22]. Tissue cholesterol and lipid contents were estimated by the methods of Crawford[23] and Folch and colleagues[24], respectively.

Hormones assays

Progesterone was assayed by an ELISA-based kit (Immuno-Technology & Steroid Laboratory Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi, India) and estradiol was assayed using an ELISA-based kit procured from Biocheck Inc, California, USA.

Fasting insulin resistance index and insulin sensitivity index

In order to evaluate insulin sensitivity for the treatment regimens, the fasting insulin resistance index (FIRI) and the insulin sensitivity index (K_{ist}) were calculated in all the experimental groups. The FIRI was calculated by the method of Kamgang and colleagues[25] and the K_{ist} was checked as per Duncan and colleagues[26].

Statistical analysis

All data are expressed as mean ± standard error and the statistical significance was evaluated using one-way ANOVA followed by Bonferroni's multiple comparisons test using Graph Pad Prism Version 3.0 for Windows, Graph Pad software, San Diego, CA, USA.

RESULTS

Body weight gain, feed intake and efficiency and water intake

Tables 1 and 2 show the changes in food and water intake, body weight gain and feed efficiency at the end of 5 weeks of treatment in sham-operated and ovariectomized animals. Compared to the sham-operated control animals, ovariectomized animals showed greater body weight gain along with 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 estradiol showed the greatest decrement in feed efficiency and body weight gain compared to all other treatments. Progesterone supplementation showed a similar trend of decrement in body weight gain and feed efficiency.

Table 1 Food and water intake in control and experimental groups. Data are given as mean ± standard error

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**	40.32 ± 3.45
OVX + E2	18.25 ± 3.12*	35.56 ± 2.56
OVX + P4	24.54 ± 2.58**	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 + E2 + ML	15.65 ± 1.11†	35.01 ± 2.56
OVX + E2 + MH	13.33 ± 1.02‡	36.00 ± 2.12

SO, sham-operated control; OVX, ovariectomized; OVX + E2, ovariectomized + estrogen; OVX + P4, ovariectomized + progesterone; OVX + ML, ovariectomized + melatonin (low dose); OVX + MH, ovariectomized + melatonin (high dose); OVX + E2 + ML, ovariectomized + estrogen + melatonin (low dose); OVX + E2 + ML, ovariectomized + estrogen + melatonin (high dose)
**, $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, †, $p < 0.01$, ‡, $p < 0.001$ when compared to ovariectomized animals

Relative organ weights

Table 3 depicts 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 ovariectomized animals compared to the sham-operated controls. This increase was significantly reversed by melatonin alone or in combination with estrogen and was much more significant compared to the decrease brought about by estrogen/ progesterone treatment individually. Ovariectomized animals showed significant decrement in uterine weight, and estrogen treatment, either singly or in combination with both doses of melatonin, showed significant increment in the uterine weights. Relative weights of liver, muscle and kidney were also increased in ovariectomized animals but were statistically insignificant.

Table 2 Body weight gain and feed efficiency in control and experimental groups. Data are given as mean ± standard error

Groups	*Body weight gain* (g)	*Feed efficiency* (body weight/100 g food intake)
SO	10.00 ± 0.21	1.62 ± 0.022
OVX	28.00 ± 0.12***	2.83 ± 0.04***
OVX + E2	12.00 ± 0.35‡	1.87 ± 0.014*
OVX + P4	8.00 ± 0.44‡	0.93 ± 0.001†,**
OVX + ML	12.00 ± 0.24‡	1.48 ± 0.002*
OVX + MH	5.00 ± 0.102***	0.82 ± 0.003***,‡
OVX + E2 + ML	8.00 ± 0.17‡	1.46 ± 0.005†
OVX + E2 + MH	5.00 ± 0.02***,‡	1.07 ± 0.004‡

For abbreviations, see Table 1
**, $p < 0.01$, ***, $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, †, $p < 0.01$, ‡, $p < 0.001$ when compared to ovariectomized animals

Table 3 Relative organ weights (g/100 g body weight) in control and experimental groups. Data are given as mean ± standard error

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***	1.75 ± 0.023***
OVX + E2	2.34 ± 0.14	0.45 ± 0.0012	1.66 ± 0.42	0.41 ± 0.02***,‡	1.24 ± 0.003‡
OVX + P4	2.32 ± 0.24	0.44 ± 0.0023	1.58 ± 0.35	0.32 ± 0.01***,‡	1.25 ± 0.0012‡
OVX + ML	2.64 ± 0.33	0.42 ± 0.0033*	1.44 ± 0.23***,‡	0.07 ± 0.002**	1.32 ± 0.045***
OVX + MH	2.77 ± 0.54	0.39 ± 0.0041*	1.51 ± 0.27	0.05 ± 0.001**	1.14 ± 0.023‡
OVX + E2 + ML	2.22 ± 0.21	0.41 ± 0.002*	1.62 ± 0.035	0.41 ± 0.02***,‡	1.22 ± 0.0024***,‡
OVX + E2 + MH	2.34 ± 0.45	0.51 ± 0.0012	1.65 ± 0.023	0.31 ± 0.01***,‡	1.23 ± 0.0052***,‡

For abbreviations, see Table 1
**, $p < 0.01$, ***, $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, ‡, $p < 0.001$ when compared to ovariectomized animals

Serum glucose, insulin and FIRI

Table 4 depicts fasting serum glucose and insulin levels along with the FIRI of all the groups. Ovariectomy did not have any significant effect on the glycemic status but showed significant increase in insulin titer compared to the sham-operated control group. There was significant insulin resistance as marked by increased FIRI in these animals. Both estrogen and progesterone treatments, although significantly reducing the serum insulin titers and FIRI values in ovariectomized animals, were not, however, successful in reverting to the control levels. 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. The combination treatment of estrogen and melatonin showed changes similar to those of melatonin alone, with E2 + ML being maximally effective.

Serum hormone profile of estrogen and progesterone

Table 5 shows the circulating sex steroid levels in all the experimental groups. Ovariectomized animals showed significant decrease in estrogen and progesterone levels, with only traces of these hormones remaining in circulation in comparison to the ovary-intact, sham-operated controls. There was significant increment in estrogen and progesterone levels in the estradiol and progesterone replacement groups.

Table 4 Fasting serum glucose, insulin and fasting insulin sensitivity index (FIRI) in control and experimental groups. Data are given as mean ± standard error

Groups	*Fasting serum glucose* (mmol/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***	2.39***
OVX + E2	5.37 ± 0.13	8.26 ± 0.24***,‡	1.774†
OVX + P4	5.62 ± 0.21	8.76 ± 0.54***,‡	1.97†
OVX + ML	5.27 ± 0.15	6.51 ± 2.5***,‡	1.37‡
OVX + MH	6.27 ± 0.03**,†	5.51 ± 0.23***,‡	1.38‡
OVX + E2 + ML	5.83 ± 0.16††,*	5.51 ± 0.12***,‡	1.28‡
OVX + E2 + MH	6.46 ± 0.05**,†	5.26 ± 1.20***, *	1.35‡

For abbreviations, see Table 1
††, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, †, $p < 0.01$, ‡, $p < 0.001$ when compared to ovariectomized animals

OGTT, IRT and insulin sensitivity index

Figure 1 and Table 6 give the results of the OGTT and Figure 3 and Table 7 give the data from the IRT in all the experimental groups. Figure 2 represents the area under the curve for OGTT and Figure 4 the insulin sensitivity index. Ovariectomy showed an increased area under the curve in comparison to the sham-operated controls. Melatonin treatment (both doses) could significantly reduce the area under the curve with an improved glucose tolerance. However, combinations of estradiol + melatonin showed maximal effect in terms of reduced area under the curve. Progesterone, however, did not show any improvement in OGTT. Except for the insulin resistance curves of OVX, OVX + P4 and OVX + MH, which were poorer compared to those of control animals, the curves of all the other experimental groups showed improvement, with OVX + E2 + ML and OVX + ML being the best in that order followed by OVX + E2 + MH and OVX + E2. The insulin sensitivity index, as represented by the

Table 5 Serum estrogen and progesterone levels in control and experimental groups. Data are given as mean ± standard error

Groups	*Estrogen* (pg/ml)	*Progesterone* (ng/ml)
SO	24.00 ± 2.12	13.90 ± 1.56
OVX	1.12 ± 0.024***	6.60 ± 0.064***
OVX + E2	80.00 ± 5.45***,‡	10.10 ± 2.11*
OVX + P4	4.23 ± 0.02	42.32 ± 3.45**,‡
OVX + ML	6.00 ± 2.11**	16.66 ± 2.84**,*
OVX + MH	5.24 ± 0.1***	13.82 ± 0.21*
OVX + E2 + ML	90.00 ± 3.87***,‡	24.23 ± 5.85***,‡
OVX + E2 + MH	85.00 ± 4.45***,‡	32.00 ± 3.58***,‡

For abbreviations, see Table 1
**, $p < 0.01$, ***, $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, ‡, $p < 0.001$ when compared to ovariectomized animals

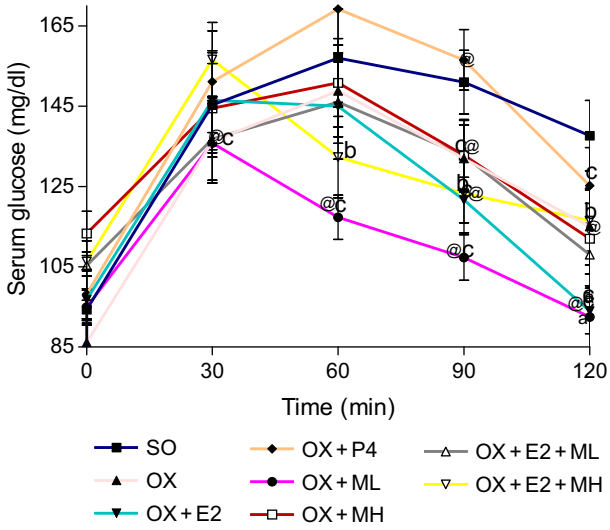

Figure 1 Glucose tolerance curves of control and experimental groups. Data are expressed as mean ± standard error. For abbreviations, see Table 1. [b], $p < 0.01$, [c], $p < 0.001$ when compared to sham-operated controls; [*], $p < 0.05$, [@], $p < 0.001$ when compared to ovariectomized animals. SO, sham-operated control; OX, ovariectomized; OX + E2, ovariectomized + estrogen; OX + P4, ovariectomized + progesterone; OX + ML, ovariectomized + melatonin (low dose); OX + MH, ovariectomized + melatonin (high dose); OX + E2 + ML, ovariectomized + estrogen + melatonin (low dose); OX + E2 + ML, ovariectomized + estrogen + melatonin (high dose)

K_{ist} values (Figure 4), also reflected the same, with maximal sensitivity being recorded for OVX + E2 + ML followed by OVX + ML, OVX + E2 + MH and OVX + E2 groups, respectively. The poorest K_{ist} values were obtained for OVX, OVX + P4 and OVX + MH groups.

Carbohydrate metabolism

Table 8 shows hepatic glycogen content and activity levels of glycogen phosphorylase and glucose-6-phosphatase. Ovariectomized animals showed significant decrease in hepatic glycogen content and significant increment in the activity levels of both enzymes. In general, except for OVX, OVX + P4 and OVX + MH groups of animals, which showed similar changes of decreased glycogen content and increased phosphorylase and glucose-6-phosphatase activities, all other groups of animals effectively reversed the OVX-induced changes in the order E2 > E2 + ML > ML > E2 + MH. Table 9 shows the muscle glycogen content along with phosphorylase activity. Ovariectomy significantly decreased the muscle glycogen content and increased glycogen phosphorylase activity as compared to sham-operated controls. Similar to the hepatic changes, even muscle glycogen content and phosphorylase activity were the poorest in the OVX, OVX + P4 and OVX + MH groups of animals. The most favorable changes in terms of reversal of OVX-induced changes were seen in ML followed by E2, E2 + ML and E2 + MH groups.

Table 6 Rates of elevation and clearance of glucose during glucose tolerance test in control and experimental groups

Groups	*Rate of elevation*	*Rate of clearance*
SO	1.21	0.488
OVX	0.83	0.266
OVX + E2	1.65	0.583
OVX + P4	1.83	0.730
OVX + ML	1.33	0.588
OVX + MH	0.77	0.450
OVX + E2 + ML	0.67	0.633
OVX + E2 + MH	2.01	0.446

For abbreviations, see Table 1

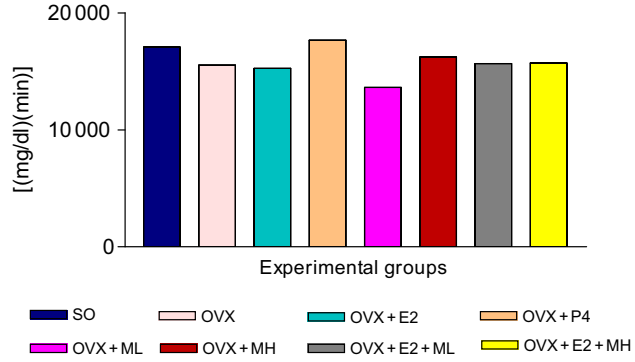

Figure 2 Area under curve for glucose tolerance test in control and experimental groups. For abbreviations, see Figure 1

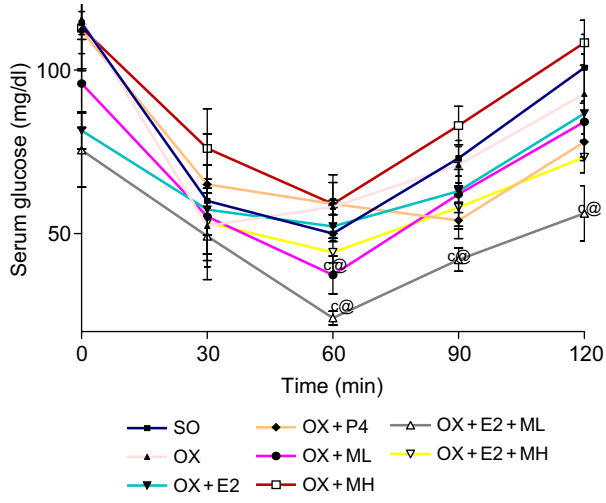

Figure 3 Insulin response curves in control and experimental rats. Data are expressed as mean ± standard error. For abbreviations, see Figure 1. [a], $p < 0.05$, [b], $p < 0.01$, [c], $p < 0.001$ when compared to sham-operated controls; [*], $p < 0.05$, [e], $p < 0.01$, [@], $p < 0.001$ when compared to ovariectomized animals

Table 7 Rates of clearance and elevation of glucose during insulin response test in control and experimental groups

Groups	*Rate of clearance*	*Rate of elevation*
SO	1.07	0.844
OVX	2.1	1.220
OVX + E2	0.80	0.575
OVX + P4	0.64	0.800
OVX + ML	0.68	0.319
OVX + MH	1.89	0.580
OVX + E2 + ML	0.85	0.533
OVX + E2 + MH	2.64	0.322

For abbreviations, see Table 1

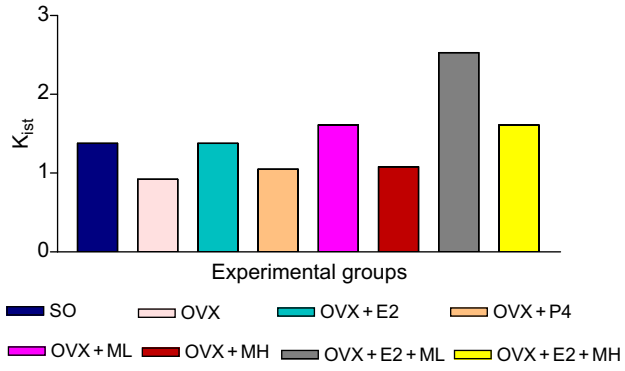

Figure 4 Insulin sensitivity index (K_{ist}) in control and experimental groups. For abbreviations, see Figure 1

Serum lipid profile

Figure 5 depicts the serum lipid profile of all the experimental groups. There was a marked increase in the serum levels of total cholesterol, triglycerides, LDL and VLDL cholesterols, with a corresponding decrease in HDL cholesterol in ovariectomized animals compared to the sham-operated controls. Both estrogen and progesterone replacement reversed the alterations observed in the ovariectomized animals, although not fully. Melatonin supplementation, both individually and in combination with estradiol, could positively regulate the changes in ovariectomized animals, with MH being maximally effective followed by E2 + MH, ML and E2 + ML in that order.

Tissue cholesterol and lipid contents

Figures 6 and 7 show the tissue cholesterol and lipid contents in all the experimental groups. Ovariectomized animals showed increment in cholesterol and lipid contents in liver, muscle and kidney. Estrogen, although significantly able to regulate the increase in tissue cholesterol content, was nevertheless less effective in reversing tissue lipid contents. Treatment with the high dose of melatonin (OVX + MH and OVX + E2 + MH) could normalize the tissue cholesterol and lipid contents most significantly. Low-dose melatonin was also efficient but less significantly as compared to the higher dose.

DISCUSSION

The present study employs ovariectomized rat 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 the estrogen-deficient state[27]. The efficacy of melatonin as a supplementation therapy (MST) has been tested as ovariectomized rats are known to exhibit increased visceral fat content, dyslipidemia, impaired glucose tolerance and defective insulin-mediated glucose clearance, as seen in insulin resistance[28]. The study evaluates the efficacy of melatonin in reversing insulin resistance and diabetogenic changes in relation to carbohydrate and lipid metabolisms, compared to that of ERT and PRT. Based on our previous studies[14,18,29], two different doses

Table 8 Hepatic glycogen content and glycogen phosphorylase and glucose-6-phosphatase activity in control and experimental groups. Data are given as mean ± standard error

Groups	*Glycogen* (mg/100 mg tissue)	*Glycogen phosphorylase* (μmol/l PO4 released/ 100 mg protein/10 min)	*Glucose 6 phosphatase* (μmol/l PO4 released/ 100 mg protein/10 min)
SO	2.42 ± 0.006	0.128 ± 0.001	0.23 ± 0.0282
OVX	2.09 ± 0.09**	0.149 ± 0.001***	0.28 ± 0.0110**
OVX + E2	2.52 ± 0.02‡	0.125 ± 0.0021‡	0.24 ± 0.0021†
OVX + P4	2.01 ± 0.012**	0.144 ± 0.0012***	0.29 ± 0.001**
OVX + ML	2.39 ± 0.004‡	0.131 ± 0.0034‡	0.22 ± 0.0341
OVX + MH	2.07 ± 0.14**	0.140 ± 0.0001**	0.26 ± 0.0203
OVX + E2 + ML	2.44 ± 0.13‡	0.120 ± 0.001‡	0.23 ± 0.0012†
OVX + E2 + MH	2.33 ± 0.11‡	0.135 ± 0.0032‡	0.235 ± 0.0022†

For abbreviations, see Table 1
**, $p < 0.01$, ***, $p < 0.001$ when compared to sham-operated controls; †, $p < 0.01$, ‡, $p < 0.001$ when compared to ovariectomized animals

Table 9 Changes in muscle glycogen content and phosphorylase activity in control and experimental groups. Data are given as mean ± standard error

Groups	*Glycogen* (mg/100 mg tissue)	*Glycogen phosphorylase* (μmol/l PO4 released/ 100 mg protein/10 min)
SO	1.02 ± 0.001	0.25 ± 0.003
OVX	0.96 ± 0.01	0.28 ± 0.001
OVX + E2	1.01 ± 0.002	0.24 ± 0.001†
OVX + P4	0.95 ± 0.0012	0.29 ± 0.002**
OVX + ML	1.11 ± 0.0014	0.23 ± 0.010†
OVX + MH	0.93 ± 0.01	0.27 ± 0.001
OVX + E2 + ML	1.03 ± 0.002	0.25 ± 0.023
OVX + E2 + MH	1.04 ± 0.01	0.27 ± 0.001

For abbreviations, see Table 1
**, $p < 0.01$ when compared to sham-operated controls; †, $p < 0.01$ when compared to ovariectomized animals

of melatonin as part of MST have been employed in the present study to assess the dose-dependent response.

MST could effectively regulate ovariectomy-induced weight gain as well as feed efficiency. These changes are seen more pronouncedly in the low-dose melatonin-supplemented ovariectomized rats than in either estrogen- or progesterone-replaced rats. Although previous findings do establish the role of ERT in reversing 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 the estrogen–melatonin combination. Our observations are at variance with the findings of Sanchez-Mateos and colleagues[30], wherein melatonin administered through drinking water had an intermediate effect in reducing body weight gain compared to estrogen or estradiol + melatonin-supplemented groups. These differential observations may be attributed to the disparity in the mode of MST 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 favorable actions of ML[31–33] as against continuous melatonin intake through drinking water which is likely to have minimal effect. The increased body weight gain seen in ovariectomized animals, also associated with increased adiposity, is nullified by MH as well as E2 + MH and E2 + ML supplementation even better than either E2 replacement or ML supplementation. The ovariectomized animals also show significant reduction in uterine weight owing to the removal of the circulating sex steroids, which is fully reversed in estrogen- and progesterone-replaced groups, in that order, as also shown by other studies[28,34]. Although E2 + ML and E2 + MH were also equally effective, MST was, however, ineffective. This ineffectiveness of melatonin therapy in recovering uterine weight is, however, of no consequence or significance in menopausal/postmenopausal women.

The increase in serum insulin titer and increased FIRI index seen in the present study are well supported by reported whole body insulin resistance and elevated insulin level in rats after 5 weeks of ovariectomy[35]. MST effects significant reduction in serum insulin levels as well as maintaining fasting glucose level and improving FIRI. Our study on MST employing two doses of melatonin given alone or in combination with estradiol reveals the former to be more effective overall than the latter (combination) in maintaining glycemic status, serum insulin titer and FIRI; in fact, both these schedules proved better than ERT. The present report is the foremost and the only one of its type to show a general efficacy of melatonin in controlling insulin resistance and fasting insulin and glucose levels in ovariectomized animals and hence provides adequate support for developing MST either alone or in combination with a low dose of estradiol as a possible alternative to ERT in postmenopausal women. Melatonin has been shown to reduce circulating insulin levels, as evidenced from our previous studies[18,29], which provide a further basis to the present contention as also do other studies[36,37]. Both OGTT and IRT reveal significantly increased area under the curve for the former and significant reduction in K_{ist} for the latter in ovariectomized animals. Another form of evaluation of insulin sensitivity, expressed as glucose–insulin (GI) index based on time-dependent changes in glucose and insulin levels under OGTT, has also reported decreased insulin sensitivity, marked by higher GI index in ovariectomized rats[28]. Impaired glucose tolerance and decreased insulin sensitivity have also been demonstrated by others in ovariectomized animals[38,39]. In the present study, neither ERT nor PRT has been potent in reversing ovariectomy-induced impairment in glucose tolerance and this finds relevance from the reports of worsened glucose tolerance with low- or high-dose oral contraceptive use[40,41] or even in postmenopausal HRT, depending on dose and type of steroid used[42]. However, the response to exogenous insulin in the IRT does show a significant degree of normalization in ERT rats not observed in PRT, as also evidenced by the report from Kumagai and colleagues[38] in which estrogen treatment restored insulin sensitivity and progesterone treatment resulted in insulin resistance. Melatonin administration, either alone or in combination with estrogen, does register better glycemic regulation by improving glucose tolerance with less area under the curve and by improving insulin sensitivity, as seen from the K_{ist} value. Of all the MST groups, OVX + E2 + ML shows the most effective response in terms of both glucose tolerance and insulin sensitivity, with OVX + ML, OVX + E2 + MH and OVX + E2 being the next in their order of efficacy. In this context, the effectiveness of melatonin in maintaining glucose homeostasis in diabetic and non-diabetic animals has been reported from our laboratory and also by others[18,29,43,44]. Apparently, the present findings highlight the fact that MST with a low dose is much more effective than ERT in improving insulin sensitivity, although MST in combination with E2 has the maximal effect.

Impaired carbohydrate metabolism in ovariectomized animals is indicated by decreased hepatic and muscle glycogen content with a concomitant increase in phosphorylase and glucose-6-phosphatase activities, changes that are diabetogenic[45]. Ovariectomized animals, as a model for mild obesity and insulin resistance, are characterized by reduced

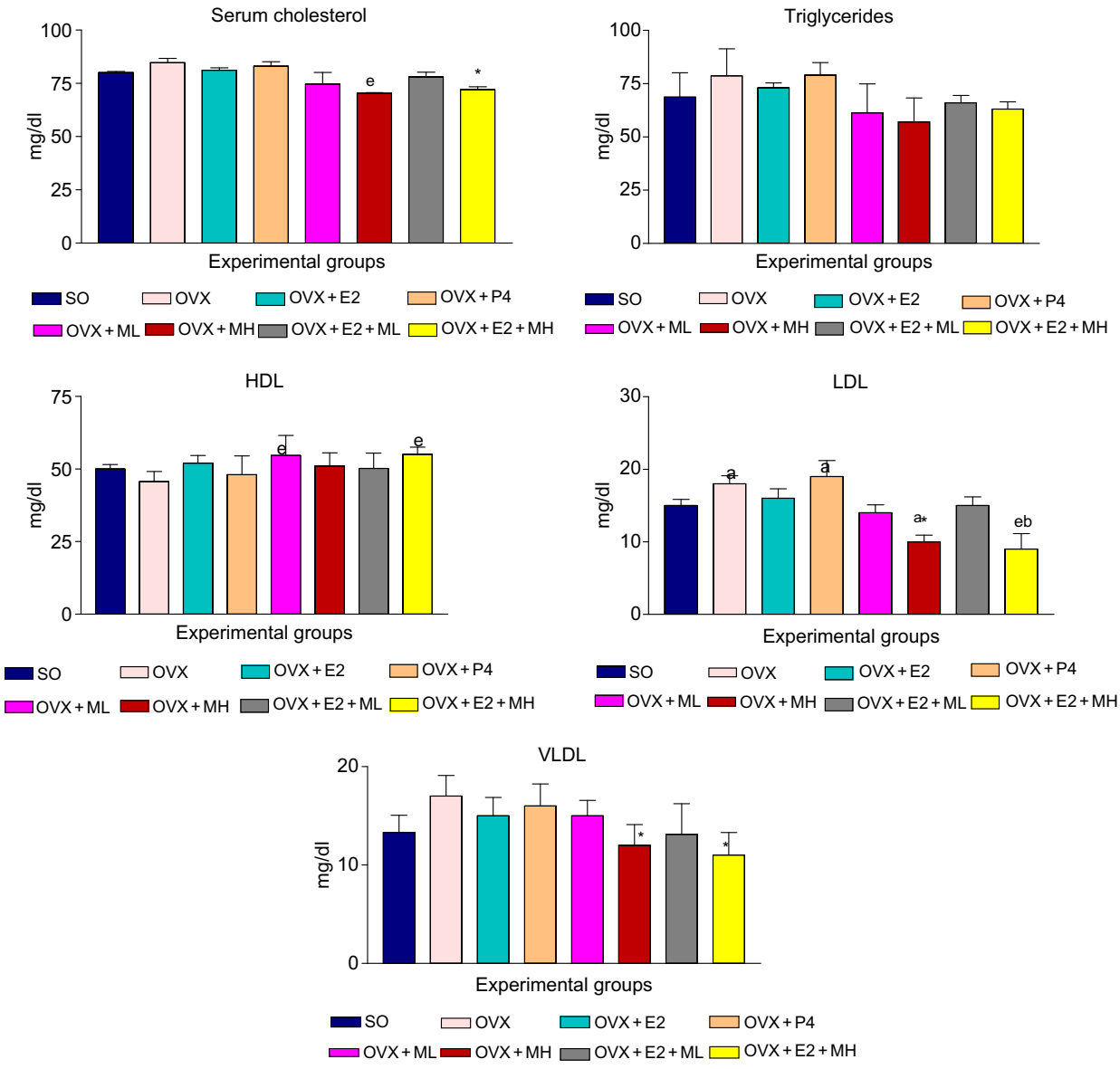

Figure 5 Changes in serum lipid profile in control and experimental groups. Data are expressed as mean ± standard error. For abbreviations, see Figure 1. [a], $p < 0.05$, [b], $p < 0.01$, [c], $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, [e], $p < 0.01$, [@], $p < 0.001$ when compared to ovariectomized animals

glycogen content[45], and impaired insulin-stimulated glycogen synthesis in the insulin-resistant state has also been reported[46]. Estrogen, but not progesterone, is seen to be effective in reversing the ovariectomy-induced changes in tissue glycogen contents and activities of phosphorylase and glucose-6-phosphatase. In animals subjected to MST, ML appears 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[17]. A mechanistic explanation for the observed decrease in tissue glycogen contents and insulin resistance could be an ovariectomy-induced reduction in expression of the glucose transporter GLUT 4 in peripheral tissues and consequent compromised glucose uptake. Evidence to this end is available from the works of Saengsirisuwan and colleagues[28], showing reduced muscle GLUT 4 expression ovariectomized animals and its recovery upon estradiol replacement, and of Barros and colleagues[47], demonstrating insulin resistance by way of GLUT under-expression in estrogen receptor knockout mice (ERα−/−). Our demonstration herein of reduced insulin resistance in ovariectomized 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[29]. Apparently, melatonin supplementation therapy is competent enough to restore glycemic

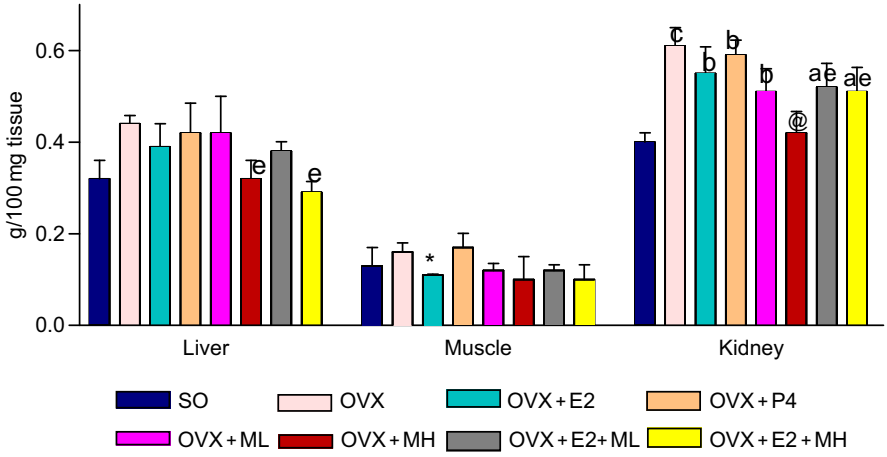

Figure 6 Tissue lipid content in control and experimental groups. Data are expressed as mean ± standard error. For abbreviations, see Figure 1. [a], $p < 0.05$, [b], $p < 0.01$, [c], $p < 0.001$ when compared to sham-operated controls; *, $p < 0.05$, [e], $p < 0.01$, [@], $p < 0.001$ when compared to ovariectomized animals

dysregulation, insulin resistance and reduced glucose uptake brought about by estrogen deficiency and could be thought of as an alternative to estradiol.

Along with impairment in carbohydrate metabolism, dysregulation of lipid metabolism is also indicated by the observed hyperlipidemia, 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 ovariectomized rats used as a model for understanding postmenopausal changes[28,48]. Of the various replacement/supplementation therapies employed in the present study, MH and E2 + MH are maximally effective in rectifying the ovariectomy-induced lipidemia, cholesterolemia and adiposity followed by ML, E2 + ML and E2 in that order. It is interesting to note that MST with ML is equally effective or even better than ERT in correcting ovariectomy-induced dyslipidemia and tissue lipid load. This observation is contradictory with the report of no significant effect of melatonin in ovariectomized rats[30]. 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 vs. continuous), as discussed earlier, seems to best explain the observed discrepancies. Antihyperlipidemic and antihypercholesterolemic effects of melatonin have been well documented in both experimental animals and in humans[49,50]. The present findings are further embellished by our previous studies consistent with melatonin-mediated regulation of dyslipidemia[51].

In conclusion, our data taken as a whole, without looking into individual parameters, suggest dose-dependent efficacy of melatonin as a supplementation therapy in controlling ovariectomy-induced insulin resistance and dyslipidemia. In terms of need for total exclusion of estradiol (given its risks), this study provides evidence for the potent effectiveness of MST compared to ERT as it is able to revert the ovariectomy-induced changes, except for uterine weight, and thereby compensate for sex steroid deficiency without any attendant side-effects. An intermediate dose, in between the two doses

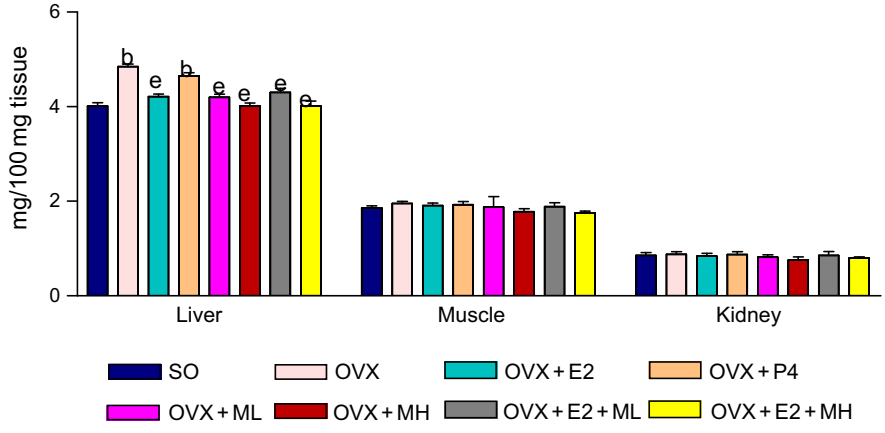

Figure 7 Tissue cholesterol contents in control and experimental groups. Data are expressed as mean ± standard error. For abbreviations, see Figure 1. [b], $p < 0.01$ when compared to sham-operated controls; [e], $p < 0.01$ when compared to ovariectomized animals

employed in the present study, may be an ideal dose to evaluate in order to combat all ovariectomy/postmenopausal changes, in keeping with the observed differential effects on carbohydrate and lipid metabolisms.

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