DIABETIC OXIDATIVE STRESS AND HEPATIC AND RENAL DYSFUNCTIONING IN ESTROGEN DEFICIENT RATS: RELATIVELY GREATER EFFICACY OF MELATONIN OVER ERT IN REMEDIATION

Maintenance of a critical balance between pro-oxidant and antioxidant mechanisms of the body defines normal physiology and, any condition that disturbs this crucial balance, can predispose the body to various pathophysiological manifestation, by the resultant increased oxidative stress (Valko *et al.*, 2004; Ozbasar *et al.*, 2010). Appropriate hormonal balance is one of the crucial determinants of normal functioning and an upset in this balance can contribute to altered pro-oxidant to antioxidant status as well as disturbances in metabolic processes (Kankofer *et al.*, 2007). Natural or surgical or medical menopause in women results in a physiological condition of the ovarian hormone deficiency. Such a state is by now well established to increase the generation of reactive oxygen species and contribute to the pathogenesis of various diseases (Kankofer *et al.*, 2007; Enli *et al.*, 2009). Ovarian hormone depletion as achieved by ovariectomy in rats is one of the most commonly used models to study the metabolic alterations associated with menopause in women (Xu and Yu, 2002; Kankofer *et al.*, 2007; Enli *et al.*, 2009).

One of the most common disorders associated with post menopausal women (PMW) is diabetes. It is also well documented that oxidative stress is both cause and consequence of diabetes (Bonnefont-Rousselot *et al.*, 2000; Maritim *et al.*, 2003). This suggests that increased oxidative stress in non-

diabetic PMW can be a predisposing factor for development of diabetes and may be equally detrimental for post menopausal diabetic women by further fanning the progression of diabetic complications.

In this context, role of hormone replacement therapy in protecting against oxidative damage in PMW is critical as a decline in estrogen is believed to be related to free radical generation in PMW (Kankofer *et al.*, 2007). There are reports wherein estrogen replacement at physiological and supraphysiological levels have been shown to provide antioxidant activity and yet other reports suggesting the protective action of estrogen against oxidative injury in liver, thereby attending to the antioxidant properties of estrogen (Muthusami *et al.*, 2005; Sener *et al.*, 2005). In this context, ERT stands justified as an effective therapy as an antioxidant in PMW and causes of POF/surgical menopause, as E_2 has positive impact on antioxidant systems (Sener *et al.*, 2002; Oztekin *et al.*, 2007) but, there are enough reported evidences to suggest undesirable effects and complication much less undisputable control of oxidative stress, thereby casting serious doubts on the validity of ERT as an unambiguous safe mode of application to women (McManus *et al.*, 1997; Santanam *et al.*, 1998; Bureau *et al.*, 2002; Harman, 2006; Arnal *et al.*, 2007; Bell and Bloomer, 2010). The reported real misgivings and uncertainties regarding ERT make it meaningful and need of the hour to search for other more acceptable alternatives to ERT in treating symptoms and complications of menopause (natural, premature or induced) compounded with diabetes as E_2 deficiency alongwith diabetes is known to be

more serious in terms of oxidative stress and other related complications (Albayrak *et al.*, 2010; Ulas and Cay, 2010).

Melatonin is a potent hydroxyl and peroxyl radical scavenger (Tan *et al.*, 2003; Hardeland, 2008) and as a pineal hormone, its major function is regulation of circadian changes but, more diversified potential for this hormone as high levels of melatonin are maintained in various organs not commensurate with its availability from pineal alone (Tan *et al.*, 2003; Carrillo-Vico *et al.*, 2004). It also possesses an important ability to diffuse through membranes and thereby regulate expression of several antioxidants and pro-oxidant enzymes at the genic level (Samuelssob *et al.*, 1987; Pozo *et al.*, 1994; Barlow-Waldeln *et al.*, 1995). There are several studies that have evidenced protective effect of melatonin against β cell damage and subsequent development of Type I diabetes in experimental diabetic rats (Montilla *et al.*, 1998; Aksoy *et al.*, 2003; Gorgun *et al.*, 2004).

The present investigation is designed to elucidate the potentials of melatonin supplementation (MST) in a dose dependent manner on various oxidative stress parameters, markers of hepatic and renal dysfunction and serum corticosterone level in ovariectomized diabetic rats, used as a model to understand the potential risk of hyperglycaemia and estrogen deficiency existing together in terms of generation of oxidative stress, a common feature characteristic of women who either have premature ovarian failure , have undergone a surgical menopause or, are individuals in the natural menopause phase of life . The effects of MST are compared to those of estrogen replaced rats as well as a combinational treatment regimen of estrogen and

melatonin as employed in the present study. This study is unique as it makes an attempt to evaluate MST as a better alternative to ERT in combating oxidative stress and hepatic and renal dysfunctions in ovariectomized hyperglycaemic rats.

RESULTS:

Lipid peroxidation (Liver, muscle and kidney) (Table 1)

Lipid peroxidation, a major marker of tissue oxidative stress showed an increase in all the three tissues of OVX rats. Similarly, D and OVX+D groups of animals also depicted significant increase in lipid peroxidation with the latter showing the greatest increase. Consequently, out of all the treatment regimens employed, melatonin at a higher dose significantly decreased levels of lipid peroxidation in OVX+D animals while, OVX+D+ML and OVX+D+P4 showed least significant changes. On the other hand, OVX+D+E_2+MH>OVX+D+E_2+ML>OVX+D+E_2 showed relatively lesser effect in reducing levels of lipid peroxidation in that order in all the three tissues.

Non enzymatic antioxidants:

Glutathione content and Ascorbic Acid (Tables 2 and 3) (Liver, muscle and kidney)

Ovariectomy induces significant decrement in the content of both non-enzymatic antioxidants (GSH and AA). The GSH and AA contents were even further decreased in D and OVX+D animals. Out of the three tissues, hepatic ascorbic acid content was most significantly altered. High dose of melatonin

as employed in OVX+D+MH and OVX+D+E_2+MH maximally increased ascorbic acid and GSH contents with similar effect in all the tissues. Next to follow in the order of significance were OVX+D+E_2+ML and OVX+D+ML groups. OVX+D+E_2 and OVX+D+P_4 group of animals showed least significant changes in the GSH and AA contents. However, estrogen showed changes in renal ascorbic acid and muscle GSH contents similar to that observed in OVX+D+ML group of animals.

Enzymatic antioxidant status:

Catalase, GPx and SOD (Tables 4, 5 and 6) (Liver, muscle and kidney)

Both ovariectomy and diabetes led to decrease in the activities of all the three antioxidant enzymes. The activity levels of the enzymes in the three tissues were significantly decreased in OVX+D group of animals even greater than that seen in D animals. Of the treatments employed OVX+D+MH followed by OVX+D+E_2+MH and OVX+D+E_2+ML in that order showed significant increment in the activity of all the three enzymes in all the three tissues. However, melatonin low dose did show a marked increment in the activity of all the three enzymes in muscle and kidney which was comparable to that seen in the combinationa groups. OVX+D+MH>OVX+D+E_2+MH>OVX+D+E_2+ML>OVX+D+ML were able to increase the activity significantly in that order. Estrogen could also increase the activities of the enzymes although not significant enough in comparison to melatonin or melatonin+E_2 combinations.

Markers of hepatic dysfunction:

SGPT, SGOT, ALP and ACP (Table 7 and Fig 7.1, 7.2, 7.3, 7.4)

Significant increment in the activities of these marker enzymes in the serum were observed in OVX+D and D animals with the increase in the former being greater than the latter. Higher dose melatonin (OVX+D+MH) group of animals was most effective in of bringing down the activity levels to near normal. Combinational treatments (OVX+D+E_2+MH and OVX+D+E_2+ML) were the next most significant in decreasing the activity of all the four marker enzymes. Apart from the effectiveness of the above mentioned groups OVX+D+ML and OVX+D+E_2 showed similar degree of effect in decreasing the activities of these enzymes but was less significant than MH or combinations of E_2 and M. Progesterone treatment was however without any significance in the activities of the enzyme.

Markers of renal dysfunction (Urea and Creatinine) and serum corticosterone level (Table 8 and Fig 8.1, 8.2, 8.3)

Diabetic animals in both ovary intact and OVX animals showed significant increment in the levels of urea and creatinine with the increase in the latter being of greater magnitude. OVX+D+MH and OVX+D+E_2+ML are the groups where maximal significant decrement in diabetes induced increase in levels of both urea and creatinine was observed.OVX+D+E_2 and OVX+D+E_2+MH were next in order to effectively bring down the levels of these markers of kidney dysfunction. There was significant increase in the serum corticosterone titres in OVX, OVX+D and D group of animals. This increase in the corticosterone

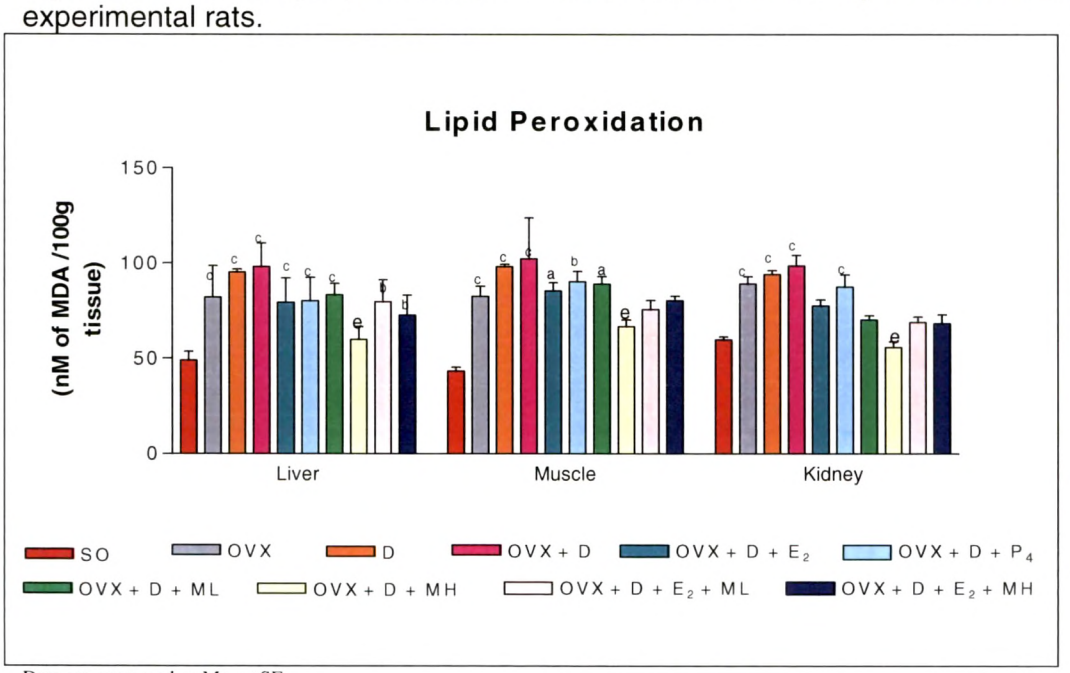
level was effectively regulated and an observed decrement was seen in OVX+D+MH, OVX+D+E_2+MH and OVX+D+E_2 groups respectively. Progesterone also showed a decrease in the serum titre of corticosterone but was less significantly reduced in comparison to the above groups and was better than melatonin at a low dose as seen in OVX+D+ML animals.

Table 1: Levels of lipid peroxidation in liver, muscle and kidney of control and experimental rats.

LIPID PEROXIDATION (nM of MDA /100g tissue)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	48.90± 4.67	43.30 ±2.190	59.70± 1.58
OVX	82.00 ±16.580^{c}	82.50± 5.280^{c}	89.20± 3.800^{c}
D	95.22± 1.550^{c}	98.23± 1.230^{c}	94.22±2.010^{c}
OVX+D	98.22 ±12.32^{c}	102.33 ±21.56^{c}	98.67± 5.650^{c}
OVX +D +E_2	79.26± 12.98^{c}	85.46± 4.36^{a}	77.54± 3.24
OVX +D +P_4	80.23± 12.27^{c}	90.23± 5.550^{b}	87.56± 6.34^{c}
OVX +D +ML	83.25± 6.100^{c}	88.95± 4.170^{a}	70.23± 2.26
OVX +D +MH	59.89± 6.800^{e}	66.58± 3.650^{e}	55.66 ±3.050^{e}
OVX +D +E_2+ML	79.66±11.490^{b}	75.68± 4.730	68.78 ±3.050
OVX +D +E_2+MH	72.56± 10.600^{b}	80.35 ±2.280	68.23 ±4.68

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<0.01when compared to ovariectomized animals.

Figure 1: Levels of lipid peroxidation in liver, muscle and kidney of control and experimental rats.



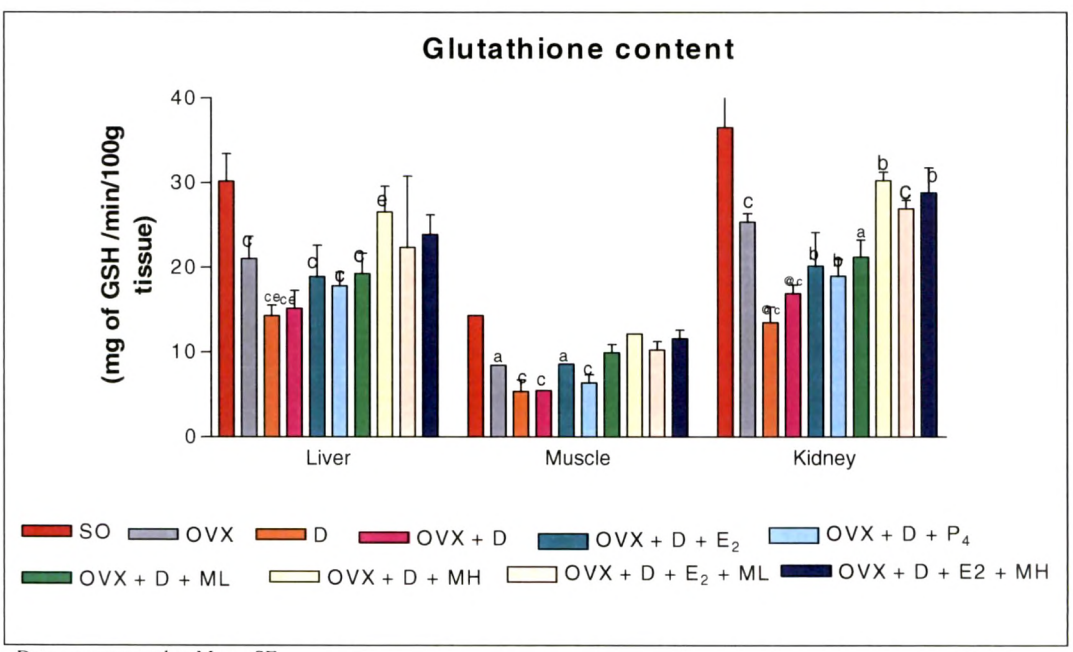
Data are expressed as Mean±SE
^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001 when compared to sham operated control and ^{e}p<0.01when compared to ovariectomized animals.

Table 2: Reduced Glutathione (GSH) content in liver, muscle and kidney of control and experimental rats

REDUCED GLUTATHIONE CONTENT (mg of GSH /min/100g tissue)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	30.15± 3.270	14.27± 0.00	36.51± 4.00
OVX	21.00 ±2.650 ^{c}	8.40 ±0.000^{a}	25.30± 1.00 ^{c}
D	14.23 ±1.280 ^{ce}	5.33 ±1.37^{c}	13.44±1.850$^{@c}$
OVX+D	15.14 ±2.210 ^{ce}	5.45 ±0.000^{c}	16.89 ±1.000$^{@c}$
OVX +D +E_2	18.88 ±3.73 ^{c}	8.56 ±0.000^{a}	20.12 ±4.00^{b}
OVX +D +P_4	17.78 ±1.640 ^{c}	6.35 ±1.000^{c}	18.95± 2.000^{b}
OVX +D +ML	19.24 ±2.430 ^{c}	9.89± 1.000	21.22 ±2.000^{a}
OVX +D +MH	26.56± 3.02 ^{e}	12.12 ±0.000	30.23 ±1.000 ^{b}
OVX +D +E_2+ML	22.35± 8.410	10.23 ±1.000	26.89±1.000 ^{c}
OVX +D +E_2+MH	23.89 ±2.330	11.56 ±1.000	28.78 ±3.000 ^{b}

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 2: Reduced Glutathione (GSH) content in liver, muscle and kidney of control and experimental rats



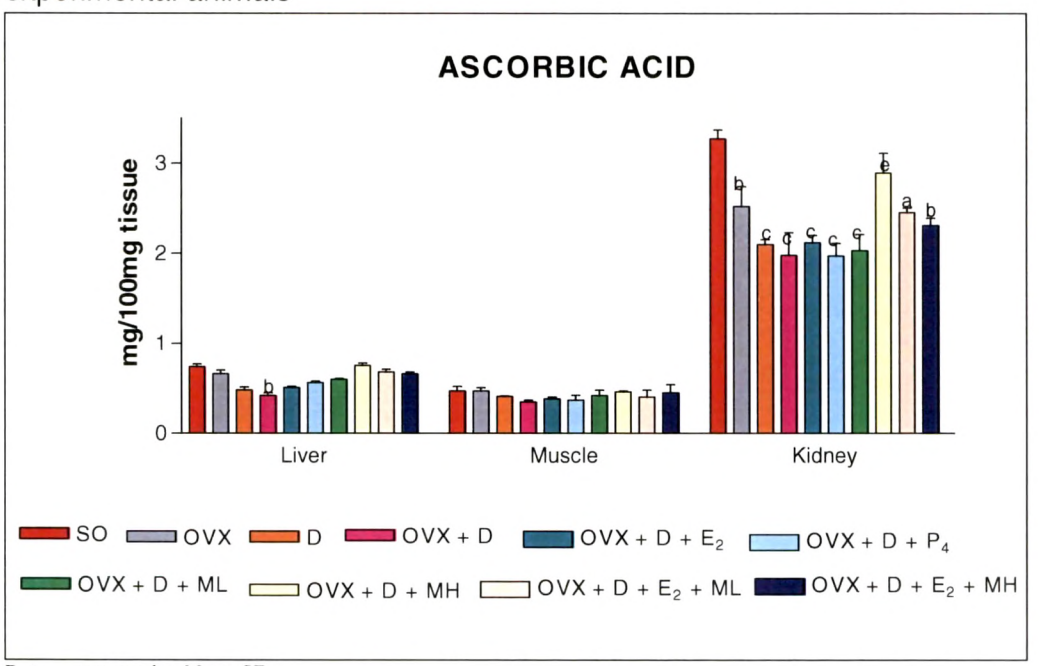
Data are expressed as Mean±SE
^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001 when compared to sham operated control and ^{c}p<0.01, $^{@}$p<0.001 when compared to ovariectomized animals.

Table 3: Ascorbic acid (AA) content in liver, muscle and kidney of control and experimental animals

ASCORBIC ACID CONTENT (mg/100mg tissue)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	0.74 ±0.030	0.47 ±0.050	3.27± 0.100
OVX	0.66± 0.040	0.47± 0.040	2.52± 0.220 [b]
D	0.48± 0.036	0.41± 0.007	2.10 ±0.050[c]
OVX+D	0.42± 0.030[b]	0.35± 0.020	1.98 ±0.250[c]
OVX +D +E_2	0.51± 0.010	0.38 ±0.020	2.12 ±0.080[c]
OVX +D +P_4	0.56± 0.020	0.37 ±0.050	1.97 ±0.140[c]
OVX +D +ML	0.60± 0.010	0.42± 0.060	2.03 0.180[c]
OVX +D +MH	0.75± 0.030	0.46± 0.010	2.89± 0.220 [e]
OVX +D +E_2+ML	0.68 ±0.030	0.40 v0.080	2.45 ±0.050[a]
OVX +D +E_2+MH	0.66± 0.020	0.45 ±0.090	2.31± 0.08[b]

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<0.01when compared to ovariectomized animals.

Figure 3: Ascorbic acid (AA) content in liver, muscle and kidney of control and experimental animals



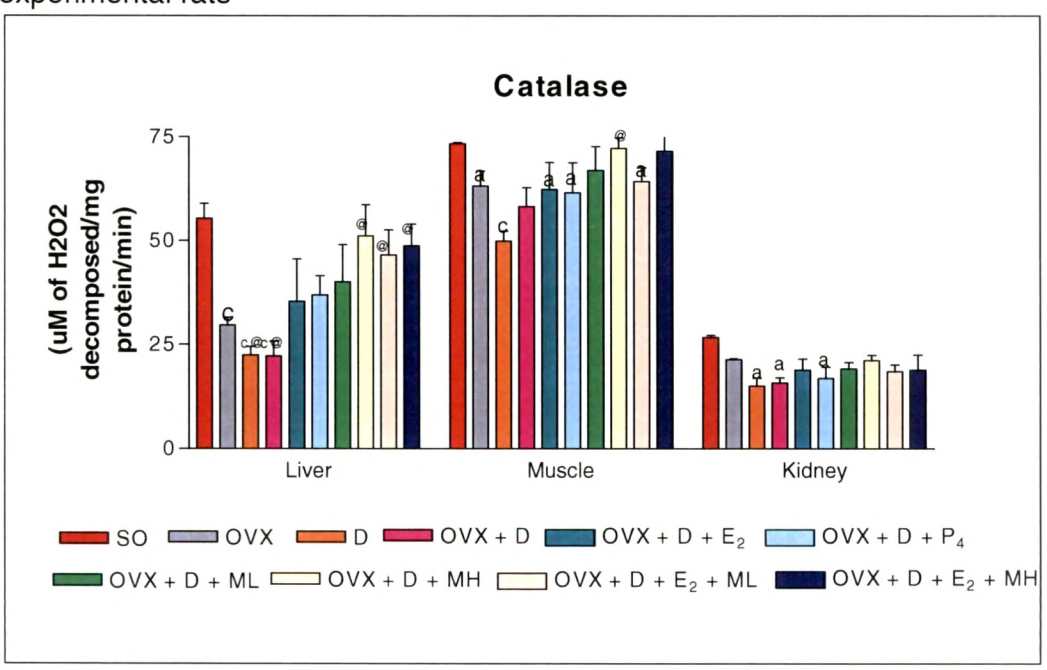
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [e]p<0.01when compared to ovariectomized animals.

Table 4: Catalase (CAT) activity in liver, muscle and kidney of control and experimental rats

CATALASE (uM of H2O2 decomposed/mg protein/min)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	55.370± 3.570	73.37± 0.220	26.74 ±0.490
OVX	29.68± 1.830^{c}	63.17 ±3.570^{a}	21.38± 0.290
D	22.45± 2.080$^{c@}$	49.92 ±2.340^{c}	15.07± 1.870^{a}
OVX+D	22.22± 3.560$^{c@}$	58.23±4.540	15.78± 1.230^{a}
OVX +D +E_2	35.34± 10.270	62.35± 6.480^{a}	18.88± 2.730
OVX +D +P_4	36.88± 4.650	61.56± 7.240^{a}	16.89± 2.780^{a}
OVX +D +ML	40.12±8.880	66.89 ±5.740	19.23 ±1.560
OVX +D +MH	51.10± 7.550$^{@}$	72.22 ±2.540$^{@}$	21.22± 1.220
OVX +D +E_2+ML	46.54± 6.060$^{@}$	64.24± 3.360^{a}	18.56± 1.570
OVX +D +E_2+MH	48.78± 5.220$^{@}$	71.55± 6.550	18.88± 3.650

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: Catalase (CAT) activity in liver, muscle and kidney of control and experimental rats



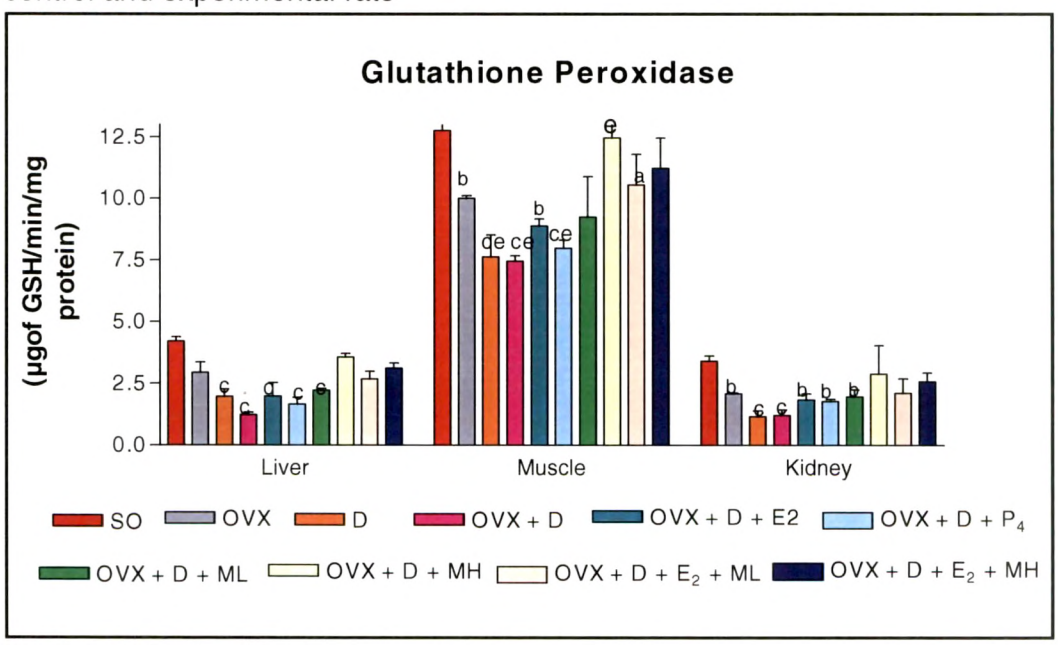
Data are expressed as Mean±SE
^{a}p<0.05, ^{c}p<0.001 when compared to sham operated control and $^{@}$p<0.001 when compared to ovariectomized animals.

Table 5: Glutathione peroxidase (GPx) activity in liver, muscle and kidney of control and experimental rats

GLUTATHIONE PEROXIDASE (µg of GSH/min/mg protein)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	4.21 ±0.170	12.76 ±0.330	3.40 ±0.210
OVX	2.94± 0.420	10.00 ±0.110 [b]	2.07± 0.050 [b]
D	1.97 ±0.302 [c]	7.63± 0.890 [ce]	1.15 0.241 [c]
OVX+D	1.23 ±0.110 [c]	7.45± 0.23 [ce]	1.21± 0.220 [c]
OVX +D +E_2	1.98 ±0.550 [c]	8.88 ±0.290 [b]	1.83± 0.250 [b]
OVX +D +P_4	1.65± 0.280 [c]	7.98 ±0.34 [ce]	1.77± 0.090 [b]
OVX +D +ML	2.21± 0.080 [c]	9.25± 1.630	1.96± 0.28 [b]
OVX +D +MH	3.56± 0.150	12.45± 1.09 [e]	2.88± 1.160
OVX +D +E_2+ML	2.68± 0.300	10.56± 1.230 [a]	2.11± 0.580
OVX +D +E_2+MH	3.12 ±0.200	11.23± 1.220	2.56 ±0.360

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<0.01when compared to ovariectomized animals.

Fig 5: Glutathione peroxidase (GPx) activity in liver, muscle and kidney of control and experimental rats



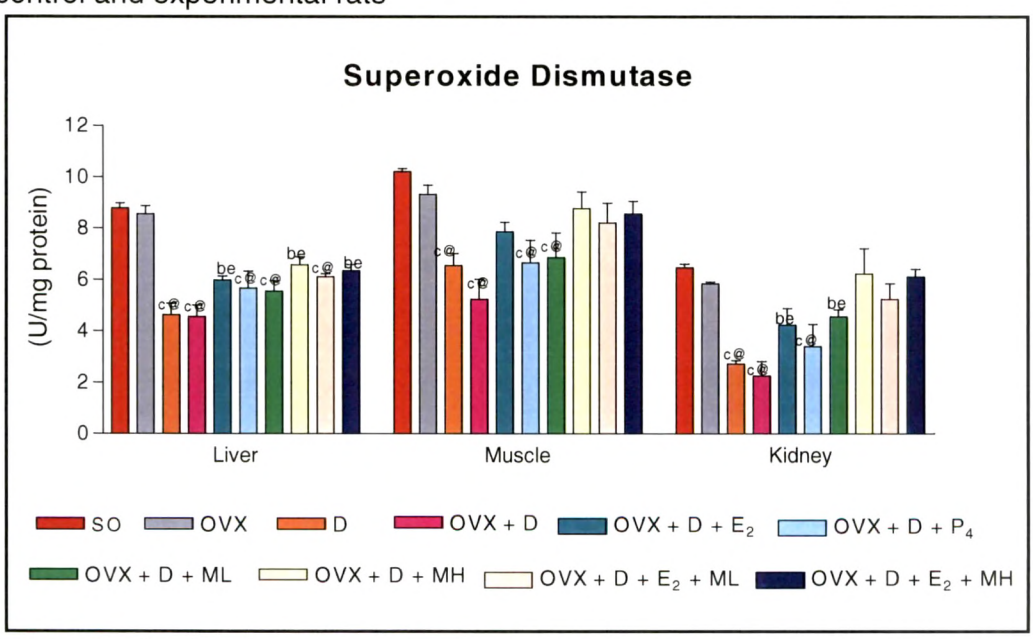
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [c]p<0.01when compared to ovariectomized animals.

Fig 6 : Superoxide dismutase (SOD) activity in liver, muscle and kidney of control and experimental rats

SUPEROXIDE DISMUTASE (U/mg protein)			
GROUPS	LIVER	MUSCLE	KIDNEY
SO	8.79± 0.180	10.21 ±0.120	6.47± 0.150
OVX	8.56± 0.310	9.33 ±0.350	5.85± 0.060
D	4.63± 0.440$^{c@}$	6.55± 0.477$^{c@}$	2.71± 0.149$^{c@}$
OVX+D	4.56± 0.450$^{c@}$	5.24± 0.78$^{c@}$	2.26± 0.560$^{c@}$
OVX +D +E_2	5.98± 0.150^{be}	7.87 ±0.37	4.23± 0.650^{be}
OVX +D +P_4	5.68 ±0.650$^{c@}$	6.66± 0.880$^{c@}$	3.39± 0.870$^{c@}$
OVX +D +ML	5.55± 0.400$^{c@}$	6.87± 0.950$^{c@}$	4.55± 0.280^{be}
OVX +D +MH	6.58 ±0.300^{be}	8.78± 0.650	6.24± 0.980
OVX +D +E_2+ML	6.12± 0.120$^{c@}$	8.21± 0.760	5.23± 0.610
OVX +D +E_2+MH	6.35± 0.25^{be}	8.56 ±0.490	6.11± 0.290

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 6 : Superoxide dismutase (SOD) activity in liver, muscle and kidney of control and experimental rats



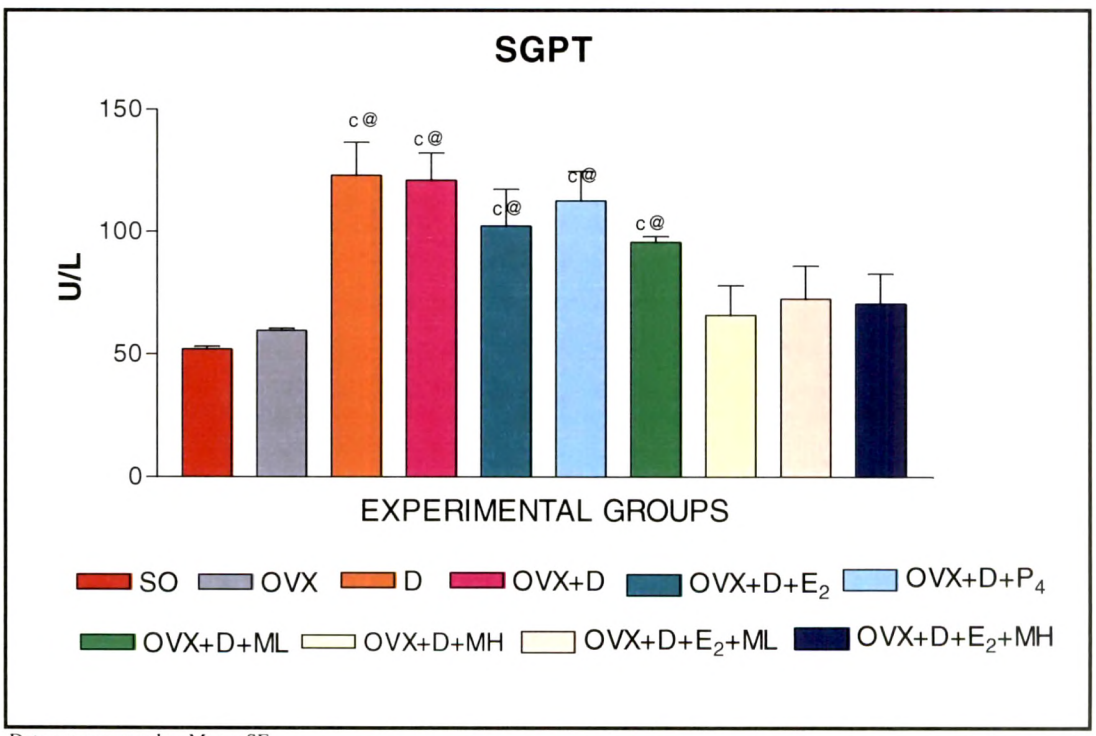
Data are expressed as Mean±SE
^{b}p<0.01, ^{c}p<0.001 when compared to sham operated control and, ^{c}p<0.01, $^{@}$p<0.001 when compared to ovariectomized animals.

Table 7: Serum markers of hepatic dysfunction in control and experimental groups.

GROUPS	SGPT U/L	SGOT U/L	ALP U/L	ACP U/L
SO	52.00±1.15	74.33±2.32	199.33±0.88	9.33±0.24
OVX	59.67±0.88	81.33±0.88	208.33±3.27	11.67±0.88
D	123.02±13.54 $^{c@}$	290.56±35.66 $^{c@}$	450.23±25.44 $^{c@}$	15.87±1.24 ^{a*}
OVX+D	121.00±11.21 $^{c@}$	210.00±12.32 ^{be}	351.00±22.56 $^{c@}$	18.89±2.12 $^{c@}$
OVX +D +E_2	102.32±15.07 $^{c@}$	185.00±21.20	288.12±22.32 $^{c@}$	15.12±1.25
OVX +D +P_4	112.56±12.15 $^{c@}$	175.23±31.45	245.56±16.33	16.24±2.38 ^{a*}
OVX +D +ML	95.68±2.33 $^{c@}$	188.56±42.66 ^{be}	267.56±32.51 $^{c@}$	15.55±3.45 ^{a*}
OVX +D +MH	65.89±12.20	146.55±22.72	208.22±12.57	10.26±1.23
OVX +D +E_2+ML	72.54±13.57	154.66±31.72	222.31±34.04	12.13±2.19
OVX +D +E_2+MH	70.54±12.30	149.25±35.20	215.56±25.59	10.21±3.44

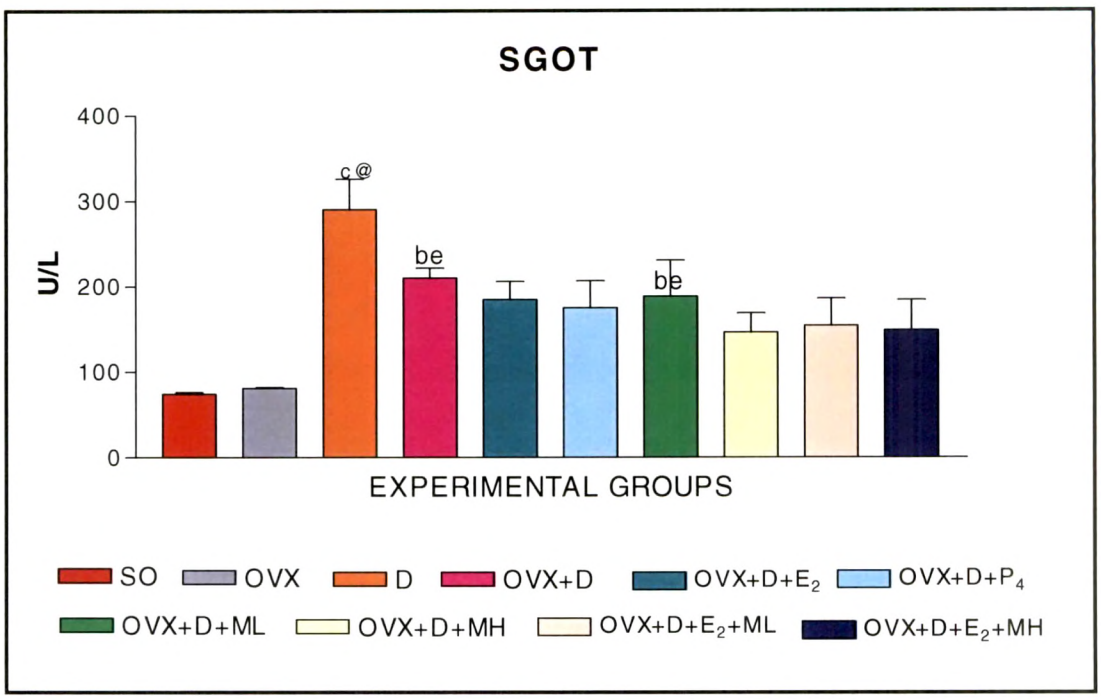
Data are expressed as Mean±SE
^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001 when compared to sham operated control and *p<0.05, ^{e}p<0.01, $^{@}$p<0.001 when compared to ovariectomized animals.

Figure 7.1: Serum SGPT activity in control and experimental groups.



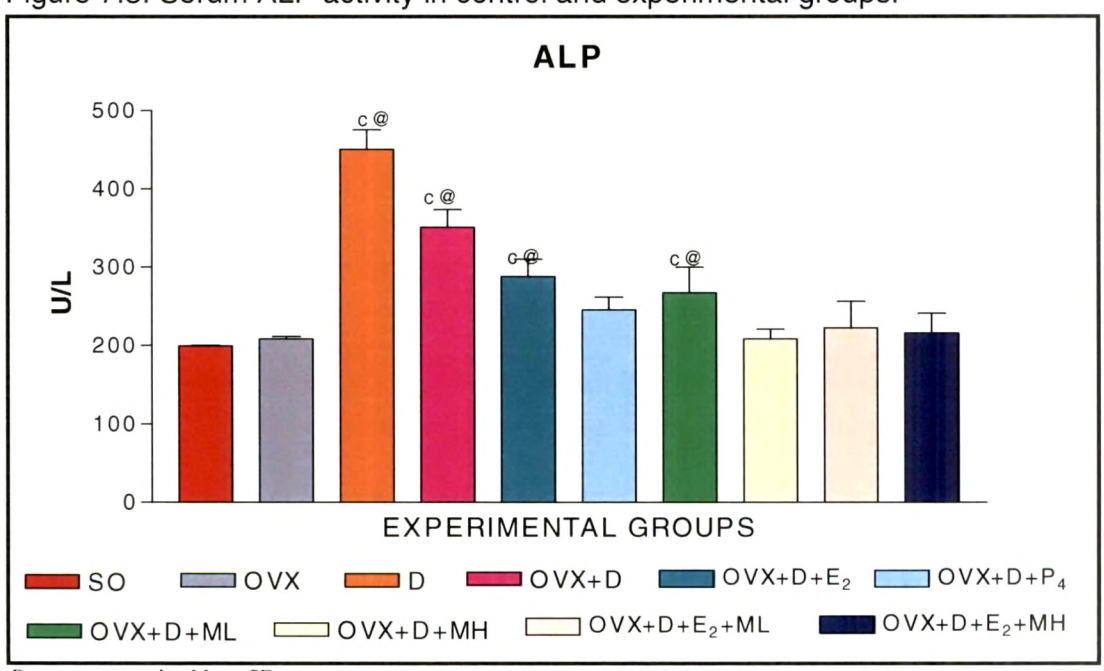
Data are expressed as Mean±SE
^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001 when compared to sham operated control and *p<0.05, ^{e}p<0.01, $^{@}$p<0.001 when compared to ovariectomized animals.

Figure 7.2: Serum SGOT activity in control and experimental groups.



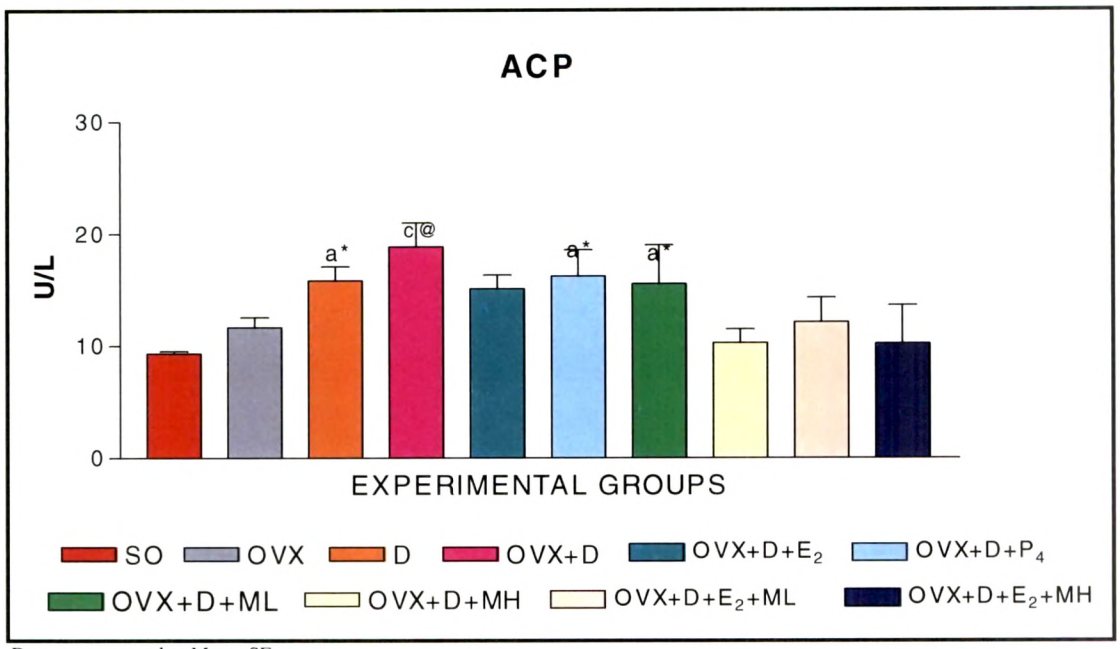
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure 7.3: Serum ALP activity 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, [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Figure 7.4: Serum ACP activity in control and experimental groups.



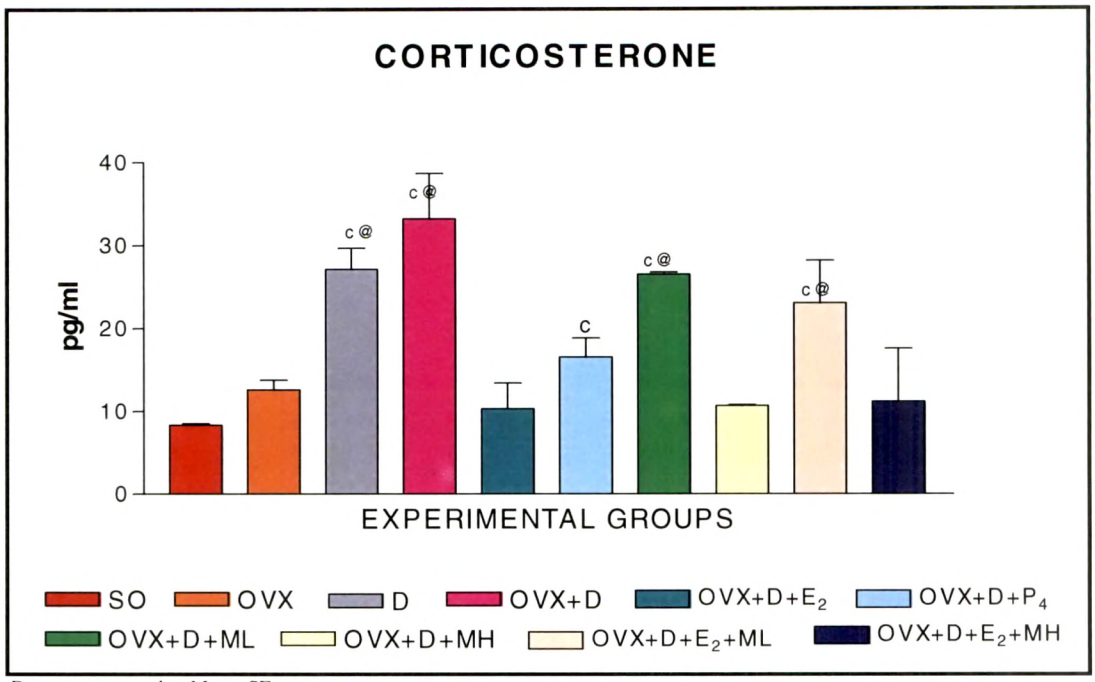
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [*]p<0.05, [c]p<0.01, [@]p<0.001 when compared to ovariectomized animals.

Table 8: Serum levels of Corticosterone, urea and creatinine in control and experimental rats.

GROUPS	CORTICOSTERONE ng/ml	UREA mg/dl	CREATININE mg/dl
SO	8.36±0.20	37.33±4.20	0.44±0.01
OVX	12.62±1.16	46.00±3.16	0.54±0.02[a]
D	27.12±2.56[c@]	154.12±12.15[c@]	0.63±0.031[b]
OVX+D	33.23±5.46[c@]	161.12±12.667[c@]	0.66±0.012[b]
OVX +D +E_2	10.30±3.11	132.11±15.45[c@]	0.55±0.02[a]
OVX +D +P_4	16.54±2.32[c]	141.00±12.35[c@]	0.58±0.01[a]
OVX +D +ML	26.50±0.26[c@]	138.33±0.68[c@]	0.61±0.02[b]
OVX +D +MH	10.70±0.07	112.24±0.58[c@]	0.52±0.02[a]
OVX +D +E_2+ML	23.00±5.18[c@]	126.33±0.78[c@]	0.53±0.01[a]
OVX +D +E_2+MH	11.20±6.35	113.25±1.99[c@]	0.51±0.01

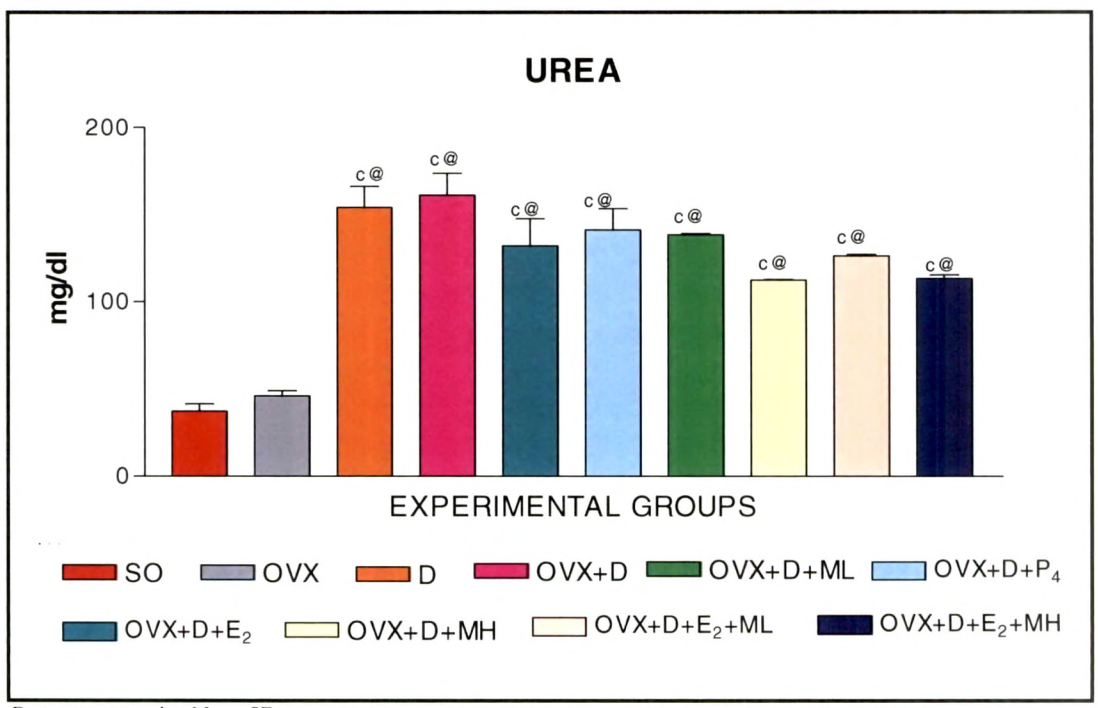
Data are expressed as Mean±SE
[a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to sham operated control and [@]p<0.001 when compared to ovariectomized animals.

Figure 8.1: Serum levels of Corticosterone in control and experimental rats.



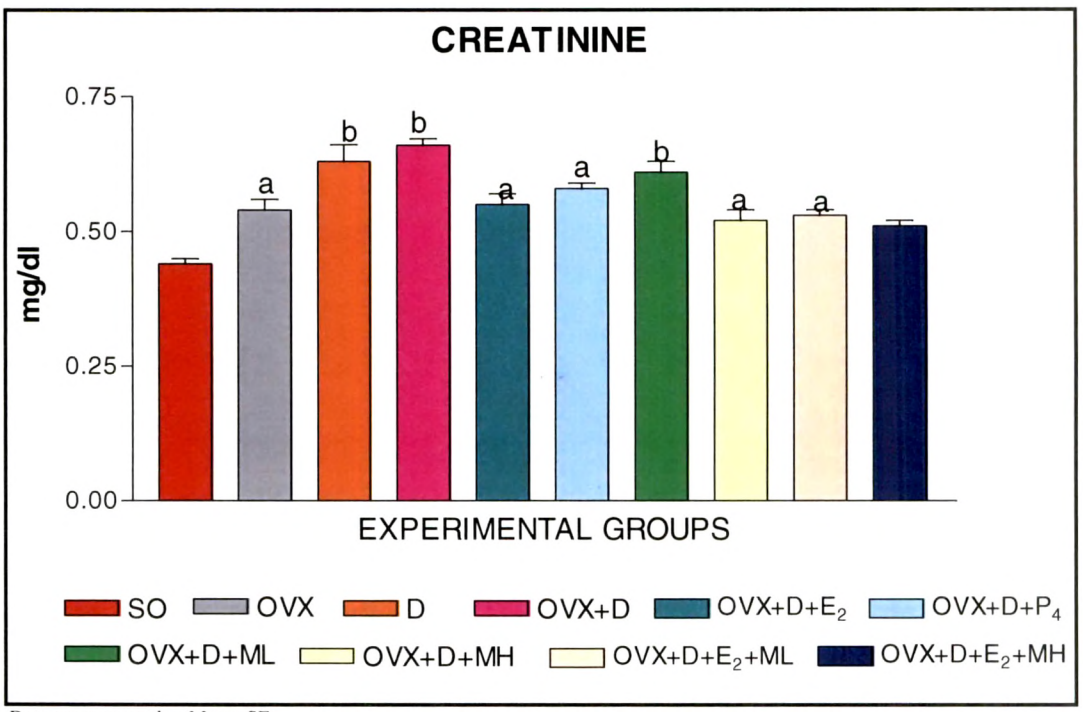
Data are expressed as Mean±SE
[c]p<0.001 when compared to sham operated control and [@]p<0.001 when compared to ovariectomized animals.

Figure 8.2: Serum levels of urea 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.

Figure 8.3: Serum levels of creatinine in control and experimental rats.



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

DISCUSSION:

Menopause is the major course of estrogen deficiency in older women but, even other physiological conditions like permanent ovarian failure , surgical removal of ovaries (surgical menopause) or medical menopause are also conditions with diminished or no estrogen in younger women. Resultant hypoestrogenic environment has been reported to escalate bone loss as one of the consequence in animals and humans (Halici *et al.*, 2008) and has also been reported to increase the risk of some neurodegerative conditions like Alzheimer's and cerebral ischemia as observed in diabetic and non diabetic menopausal females (Yong *et al.*, 2005). Ovariectomized rats are used as models to study all such pathophysiological and metabolic alterations induced due to estrogen deficiency (Turner *et al.*, 2001). Estrogen deficiency is associated with increased oxidative stress as shown by Kankofer *et al.* (Kankofer *et al.*, 2007) wherein, estrogen deficiency has been shown to alter antioxidant/oxidative parameters in rat liver and many other studies on PMW or ovariectomized rats have also recorded increased oxidative stress(Albayrak *et al.*, 2010; Ozbasar *et al.*, 2010). Hyperglycaemia as observed in Diabetes mellitus (both type I and type II) is also known to increase oxidative stress and formation of free radicals. The generated free radicals disrupt the delicate balance of pro-oxidant to antioxidant status in diabetes mellitus resulting in oxidative damage of cell components, lipids, DNA and other molecules (Naziroğlu *et al.*, 2004; Valle Gil-del *et al.*, 2005).

Hyperglycaemia and estrogen deficiency together pose a doubled risk in the generation of oxidative stress (Albayrak *et al.*, 2010, Ulas and Cay, 2010).

Hormone replacement therapy (HRT) is employed most commonly to attenuate various symptoms and disorders associated with estrogen deficiency (Greendale *et al.*, 1999). Estrogen replacement has been also reported to protect against excessive ROS generation in oxidative stress due to its inherent antioxidant properties (Kankofer *et al.*, 2007; Enli *et al.*, 2009). Recent studies on in vivo effects of estradiol as an antioxidant project a conflicting and contradictory picture by in vitro studies (Ozgonul *et al.*, 2003). Apart from this, there is also a constant debate over the beneficial role/risk of HRT in cardiovascular complications and hormone dependent cancer and hence the use of HRT in all types of estrogen deficiencies remains questionable and demands attention for better alternatives to HRT in management of menopausal symptoms. (Enli *et al.*, 2009). The present study in this behest is an attempt to evaluate the role of melatonin either singly or in combination with estrogen in reducing oxidative stress in ovariectomized diabetic rats. Melatonin is a potent hydroxyl radical scavenger and has also been associated with reduction in oxidative stress generated in streptozotocin induced diabetes mellitus (Montilla *et al.*, 1998). Apart from this, our laboratory has reported the antioxidant and antidiabetic potentials of melatonin alongwith its effect as a potent replacement agent to conventional HRT (Singh *et al.*, 2010 a,b).

Ovariectomy induced increase in LPO is clearly evident in the present study and is also well documented in literature (Hernandez *et al.*, 2000;

Baltaci *et al.*, 2004). Our study indicates a higher level of LPO in D and OVX+D groups of animals and the increase in the latter group is much higher than that seen in OVX or D animals. Increased lipid peroxidation as observed herein is indicative of a greater tissue oxidative damage in estrogen deficient hyperglycaemic state and the maximal increase in OVX+D suggest synergistic effects of both contributing to greater deterioration. Increased MDA levels in both, estrogen deficient diabetic rats and diabetic post menopausal women, have found mention in recent reports (Yong *et al.*, 2005; Ulas and Cay, 2010). There is an increasing incidence of lipid peroxidation in all the three tissues in OVX and D animals. But a generalized synergistic effect of the two is clearly evident in OVX+D group of animals.

Increased lipid peroxidation is corelatable with the levels of endogenous antioxidants. Both GSH and AA are reduced significantly in all the three tissues in OVX and OVX+D animals. Out of the three tissues the lowest GSH content is observed in muscle and the synergistic effect of both OVX and diabetes is well pronounced in all the three tissues. There are hardly any reports on muscle antioxidant level except for our earlier work which had depicted decreased GSH and AA contents in diabetic rats (Singh *et al.*, 2010a,b). In general, AA content of liver and muscle is much lesser than in kidney. No synergistic effect is visible however as the AA depletion is OVX+D animals is intermediate to those of OVX and D states. The decreased endogenous antioxidant contents recorded herein are well supported by the observations of Albayrak *et al*, (2010) in his study on liver, heart and lung tissues of Ovariectomized and diabetic ovariectomized rats.

Alongside the endogenous non-enzymatic antioxidants, enzymatic antioxidants like SOD, CAT and GPx are also potent regulators of oxidative stress. Ovariectomy induces a decrease in the activities of all the three but, the percentage decrement is even greater in the diabetic and ovariectomized diabetic groups. Tissue specific differences in the degree of decrease in enzymatic antioxidant levels are noticeable in OVX+D group of animals. Diminished activities of SOD, CAT and GPx alongwith increased lipid peroxidation have been observed in both estrogen deficient OVX rats and in diabetic rats. The decrement in the latter can be accredited to hyperglycaemia induced increase in glycation of proteins and finds support from the reported decrease in the activities of enzymatic antioxidants (Yalin *et al.*, 2006; Hamden *et al.*, 2008).

Disturbances in carbohydrate, lipid and protein metabolisms together with oxidative stress are likely to affect hepatic and renal functions in severe diabetic condition. In the present study, the changes are further potentiated in the absence of normal circulating sex steroid levels and thus believed to induce cumulative greater synergistic changes. The activities of the hepatic marker enzymes have been greatly increased in OVX+D animals in excess of that observed in OVX non diabetic animals. The activities of SGOT and ALP are tremendously increased when compared with the percentage increment in all the four enzymes under study herein. The differences however between D and OVX+D are not significant as, the changes in the activities are greater due to diabetic condition and the synergistic effect as seen for endogenous antioxidants is not evident. The urea and creatinine contents of OVX, D and

OVX+D animals show progressive increase in that order. Such reports on hepatic and renal dysfunctioning in OVX diabetic animals are rare to cite in literature and most of such changes are reported only for diabetic animals (Singh *et al.*, 2010a, b). This report is therefore a singular one on the effect of increased oxidative stress on markers of renal and hepatic dysfunction in ovariectomized and ovariectomized diabetic rats.

One of the most important markers of chronic stress in humans and animals is the serum level of cortisol/corticosterone. Increased levels of corticosterone have been reported in aged rats, which had been attributed to disturbances in HPA feedback system (Landfield *et al.*, 1978) and a similar increment in control level in aging humans is observed as well (Friedman *et al.*, 1969). We had previously reported an increase in corticosterone level in diabetic rats (Singh *et al.*, 2010a, b). In the present study, OVX+D animals have shown maximal increment in serum titres of corticosterone and this increase is greater than that seen in D or OVX animals. This increase in serum level of Cort is suggestive of increased stress status especially under conditions of estrogen deficiency coupled with hyperglycaemia.

Hormone replacement is normally attempted to ameliorate the adverse effects of sex steroid deficiency and, the present study in fact depicts a protective role of estrogen and progesterone in combating stress as such by the decrement in the circulating corticosterone titre. Estrogen and MH seem to be more potent in reversing the levels towards normalcy and the effect of estrogen is in keeping with its known action in upregulating the hippocampal adrenocorticoid steroid receptors (Ferrini *et al.*, 2005). Progesterone in

comparison is less effective in reversing the diabetes induced increase in corticosterone level thereby justifying the greater potency of estradiol in HRT compared to P_4 replacement. Both ERT and P_4 replacement could reduce lipid peroxidation almost equally in all the three tissues but then the action of estrogen is noticeably more potent than progesterone; however neither of them is able to reverse the levels totally. Certain degree of reduction in LPO seen in both diabetic and non diabetic rats subjected to ERT is justified by similar observations made by Naziroglu *et al.* (2004) and Yong *et al.* (2005). Apart from reduction in lipid peroxidation there is also considerable recovery in tissue antioxidant status. In all the three tissues, ERT shows better regulation in comparison to progesterone supplementation in increasing the endogenous antioxidant levels, though even estrogen is not able to recover the levels completely. Herein observed role of estrogen is supported by other studies on ERT and oxidative stress and the reported need for using higher doses of E_2 in combating oxidative stress (Enli *et al.*, 2009; Ulas and Cay, 2010). A higher dose is however not justified in the light of known potential adverse effects of ERT at high doses as already discussed earlier.

The present evaluation on dose dependent efficacy of MST therefore tries to explore candidature of melatonin either singly or in combination with ERT in containing the significant oxidative stress and hepatic and renal dysfunctions caused due to post-menopausal estrogen deficiency and diabetes. From the results obtained in the present study it is clear that, MST_H is able to reverse oxidative stress *in toto* and also restore the corticosterone level. MST_H is not only better than ERT but even more potent than its

combination regimen with E_2. Next in the order of effectiveness is the combination of OVX+D+ E_2+MH as it also shows almost similar changes as seen with OVX+MH. OVX+D+E_2 and OVX+ML are less effective and show more or less the same degree of efficacy. Melatonin and its role in combating oxidative stress is by now a well evidenced dictum as pinealectomized rats depict an increase in LPO and decrease in the endogenous antioxidant status which can be restored by melatonin administration successfully (Reiter *et al.*, 1999; Baydas *et al.*, 2001). Apart from the levels of endogenous antioxidants, melatonin at a high dose has also been effective in restoring the levels of hepatic and renal dysfunction markers indicating remediation of pathophysiological consequences of oxidative stress in three organs. Melatonin is also known to be potent in combating oxidative stress induced by streptozotocin and alloxan in diabetic rats very effectively at a dose of 10mg/kg body weight, as employed in the high dose MST in the present study further justifying its positive effect in reducing oxidative stress (Montilla *et al.*, 1997; Singh *et al.*, 2010b). The actions of estrogen in diabetic females is not well understood and reports obtained so far are not convincing enough to propagate its use in diabetic post menopausal women (Enli *et al.*, 2009) or in diabetic females suffering from POF or surgical menopause. In the light of reported efficacy of melatonin as a potent controller of oxidative stress, organ and DNA damage and inflammatory cytokines (Kireev *et al.*, 2008) and the present findings of superior ability of MST_H over that of ERT and even a combination of MST+ERT firmly allude to the fact that melatonin is a safe and effective alternative to ERT in alleviating estrogen deficiency and diabetes

induced cumulative aggravation of oxidative stress and tissue damage. In this context , it is worthwhile to note that both OVX induced estrogen deficiency/menopause and diabetes are characterized by spontaneous increase in inflammatory cytokines (TNF-α, IL-1β and IL-6) and diabetes in association with estrogen insufficiency augments the production of inflammatory cytokines and also aggravates oxidative injury (Kwoun *et al.*, 1997; Prediger *et al.*, 2004; Albayrak *et al.*, 2010). Apparently, increased oxidative stress and cytokines can contribute to insulin resistance and further diabetic complications affecting skeletal, renal and cardiovascular systems as, Hessen *et al.* (2002) have shown a direct influence of increased serum IL-6 on insulin resistance. There are mixed findings on the efficacy of ERT and HRT in combating OVX/menopause induced oxidative stress (Gomez-Zubeldia *et al.*, 2000; Ozgonul *et al.*, 2003; Kankofer *et al.*, 2007; Oztekin *et al.*, 2007) and is likely that a higher level of E_2 is required to fully nullify the higher oxidative stress (Bell and Bloomer, 2010); which is however beset with detrimental consequences in terms of cardio-vascular complications and cancerogenicity as discussed earlier. Added to the above uncertainties of E_2 as an efficient antioxidant in the pharmacological disadvantage need to search for alternatives that would take care of the inadequacies of ERT (Harman *et al.*, 2006; Arnal *et al.*, 2007). Melatonin fills this void as it has been identified as a powerful antioxidant cum anti-inflammatory agent under different experimental and natural conditions (Oztekin *et al.*, 2007; Kireev *et al.*, 2008) that is safer and well tolerated even in higher doses and also has the ability to cross blood-brain barrier (Holliman *et al.*, 1997; Feng and Zhang,

2005). The present findings complainant these and provide compelling evidence for consideration of MST at a slightly higher dose than what was required to combat dysglycaemia and alterations in carbohydrate metabolism as shown by us previously (Chapter 1).

It is concluded from the present evaluation that MST is a safer and effective alternative to ERT in combating the augmented oxidative stress and associated complications that befall PMW or younger women suffering from POF or subjected to surgical menopause and also suffering from diabetes.