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Effect of *Pleurotus Ostreatus* on the endothelial dysfunction in ovariectomized- diabetic rats

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We designed this study to evaluate the effect of *Pleurotus Ostreatus* mushroom on the endothelial dysfunction in ovariectomized-diabetic rats. In this study 70 female Sprague-Dawley rats were divided into 7 groups: control group, healthy rats received orally 100mg/kg body weight mushroom, healthy rats received orally 200mg/kg body weight mushroom, Sham group, ovariectomized-diabetic rats, treated ovariectomized-diabetic rats received orally 100mg/kg body weight mushroom and treated ovariectomized-diabetic rats received orally 200mg/kg body weight mushroom. Blood samples were collected for assessment of fasting sugar, insulin, estrogen, lipid profile, paraoxonase (PON1) and asymmetric dimethylarginine (ADMA). Aorta tissues were used to determine nitric oxide (NO) and tissue plasminogen activator t-PA. Treatment with *Pleurotus Ostreatus* showed a significant decrease in levels of fasting sugar, estrogen, total cholesterol (TC), triacylglycerol (TG), low density lipoprotein cholesterol (LDL-C), and ADMA. Otherwise, insulin, high density lipoprotein cholesterol (HDL-C), PON1, NO and t-PA were significantly increased compared to ovariectomized-diabetic rats. In conclusion, it has been demonstrated that *Pleurotus Ostreatus* has favorably effects on serum lipids, improves endothelium-dependent vasodilatation, increase estrogen level which decrease intravascular thrombus propagation. These findings have generated proposed mechanisms for *Pleurotus Ostreatus* to improve endothelial dysfunction and protect against the development of atherosclerosis in ovariectomized-diabetic rats.

Keywords: *Pleurotus Ostreatus*, endothelial dysfunction, estrogen, tissue plasminogen activator, paraoxonase.

INTRODUCTION

Endothelial dysfunction is a systemic disorder result from several disease processes as in diabetes mellitus, hypercholesterolemia, menopause, and hypertension (Arya et al. 2016). The imbalance between the production of vasoconstrictor and vasodilator endothelium-derived factors are the main cause of endothelial dysfunction. This imbalance occurs as a result generation of reactive oxygen species (ROS) which reduced bioavailability of NO and inactivate

endothelial cells (Salisbury & Bronas 2015). Lowering in vascular NO may be associated with diabetes through decrease synthesis of prostacyclin that results in a vasoconstriction (Vanhoutte, 2011).

The uncoupling of endothelial nitric oxide synthase (eNOS) and reduction of NO production causing by the increase of oxidative tetrahydrobiopterin (BH₄), a cofactor that regulates NO release (Hamilton and Watts., 2013). In addition increased levels of asymmetric

dimethylarginine (ADMA), the main endogenous inhibitor of eNOS through competition with L-arginine, may further reduce NO release (Sibal et al. 2010).

In diabetic patients, atherosclerosis is multifactorial with a very complex interaction including hyperglycemia, hyperlipidemia, oxidative stress, hyperinsulinemia and alterations in coagulation and fibrinolysis (Ding et al. 2005), leading to impair endothelial function. Increasing of insulin resistance with diabetes mellitus is associated with an increased free fatty acids (Taskinen., 2005) as well as increased plasma plasminogen activator inhibitor-1 (PAI-1) or decreased tissue plasminogen activator (t-PA) that inhibiting fibrinolytic activity (Hadi and Suwaidi., 2007) thus lead to endothelial dysfunction and cardiovascular events.

Menopause is known as the continues cessation of menstruation as result to loss of ovarian-follicular vitality (Bhavsar et al. 2016), with a decrease in the levels of estrogens affecting many tissues of the body (Al-Azzawi and Palacios.,2009). Estrogen deficiency causes endothelial dysfunction which is mediated through increasing production of ROS, modification of lipid profile (El Habachi et al. 2014) and lower endothelial nitric oxide synthase (eNOS) expression and activation by a related decrease in estrogen receptors (Gavin et al. 2009). Postmenopausal diabetic women are at higher risk for incidence endothelial dysfunction. They suffer from increased cardiovascular risk factors as a result of increase in their atherogenic lipid profile, and redox imbalance (El-Nasr et al. 2011). Hormone replacement therapy (HRT) has been used to relief menopause symptoms. However, due to the possible side effects of HRT, there is a growing demand for alternatives for the treatment of pathological processes and symptoms associated with menopause (Miquel et al. 2006).

Mushroom *P. Ostreatus*, belongs to the family Pleurotaceae, is valuable health food, low calories and fats contains high levels of fiber, crude polysaccharide, lovastatin, polyphenolic compounds such as phytoestrogen and flavonoids, vitamin A,C and E, β -carotene and selenium. These ingredients have created suggested mechanisms for these findings including, inhibition platelet aggregation, increased antioxidant activity, reduction of total cholesterol and low-density lipoprotein cholesterol concentration, alleviating heart disease and reducing of blood glucose levels (Jayakumar et al. 2011).

This study was aimed for determination the capability of *Pleurotus Ostreatus* in improving biochemical changes of endothelial dysfunction initial event in progress of atherosclerosis in ovariectomized-diabetic rats.

MATERIALS AND METHODS

Preparation of the mushroom extract:

Fresh oyster mushrooms were lyophilized by freeZone[®] 4.5 Liter Freeze Dry Systems at temperature (-52°C) and vacuum 0.01 mbar and then ground. The grounded extract was then converted to nano particles by PQ-N₂ Planetary Ball Mill with 100 grains 0.7cm and 2 grains 1.5cm at 45000 rpm for 30 minutes and then dry biomass was stored at 4°C. The ground materials were then re-suspended in distilled water at the concentration 18mg/ml and incubated overnight at 4°C. The suspension was centrifuged at 7000 rpm (5200 g) for 10 minutes, and the supernatant was filtered through a 0.22 μ m filter (Jedinak et al. 2011).

Ovariectomy and Sham operation:

Under general anesthesia using ether, the rats were bilaterally ovariectomized by dorsal approach. A single longitudinal skin incision was done on the dorsal midline at the level of the kidneys. The ovaries were removed after ligating the uterine horns. In sham groups rats were operated during which the ovaries were exposed but left intact (Mattila et al. 1998).

Induction of diabetes:

Streptozotocin (STZ) was purchased from sigma chemical co.(St. Louis, Mo, U.S.A) and dissolved in 50 mM sodium citrate solution (pH 4.5) containing 150 mM sodium chloride (Kohli et al. 2004).

The solution (6 mg/100 g body weight) was subcutaneously administered in rats. Fasting blood sugar was estimated after 3 days to confirm the development of diabetes mellitus. The animals were considered diabetic if fasting glucose level was \geq 200mg/dl.

Animals

Seventy Female Sprague-Dawley rats with average age 10 months and 200-210g weight were supplied by the animal house of the National Research Centre, Giza, Egypt. All rats were housed individually in stainless steel cages for two months and fed standard rodent chow. They were kept in standard conditions of temperature and

light for two weeks as an acclimatization period. The present study was approved by the Ethical Committee of the National Research Center (NRC), Egypt, which provided that the animals will not suffer at any stage of the experiment.

Animals were divided into 7 equal groups (each 10 rats) as follow:

Group I: Healthy rats served as control.

Group II: Healthy rats receiving 100mg/kg body weight mushroom for by oral gavage 45 days (Ghaly et al. 2011)

Group III: Healthy rats receiving 200mg/kg body weight mushroom by oral gavage for 45 days (Ghaly et al. 2011)

Group IV: Sham group.

Group V: Ovariectomized - diabetic rats (OVX-STZ).

Group VI: OVX-STZ- rats receiving 100mg/kg body weight mushroom by oral gavage for 45 days (Ghaly et al. 2011) after two months of the ovariectomy (Gortan et al. 2013).

Group VII: OVX-STZ- rats receiving 200mg/kg body weight mushroom by oral gavage for 45 days (Ghaly et al. 2011) after two months of the ovariectomy (Gortan et al. 2013).

Biochemical Analysis

After the experimental period, animals were kept fasting for 12 h, then anesthetized under light ether anesthesia. Blood samples collected from dorsal aorta were placed in three tubes; the first contains sodium fluoride for determination of fasting blood sugar, the second contains anti-coagulant (heparin) for plasma separation and the last was dry clean tube for serum separation. All tubes were centrifuged at 3000 rpm using cooling centrifuge (Laborzentrifugen, 2K15, Sigma, Germany) for 10 min. Plasma and sera were stored at -30°C until use.

Preparation of tissue:

Aorta tissue samples (100 mg/ml buffer) were homogenized in 50 mM phosphate buffer (pH 7.0), and then centrifuged at 10,000 rpm for 15 min; the supernatant was used for measurement of nitric oxide and tissue plasminogen activator.

Biochemical assays

Fasting plasma glucose was done immediately by the glucose oxidase method (BioMerieux, Marcy l'Etoile, France) according to Passing and Bablok, 1983. TC, HDL-C, LDL-C and TAG were measured by the colorimetric enzymatic assays using kits supplied from Biocon Diagnostic (Germany) according to Allain et al. 1974, Lopes-

Virella et al. 1977, Friedewald 1972 and Glick et al. 1986 respectively. Serum PON1 was measured spectrophotometrically according to the method of Hussein et al. 2013. Tissue nitrite/nitrate (NOx) was measured using ELISA microplate readers by the modified Griess method according to Tatsch et al. 2011. Serum insulin level was determined using ELISA (DRG, USA.) according to the method of Judzewitsch et al. 1982. The concentration of plasma estrogen and t-PA were determined by ELISA Kit (DRG Instruments, Beijing, China). ADMA was determined by HPLC method modified from the method described previously (Kurt et al. 2012).

Statistical analysis:

Data analysis was done using the statistical package for the social sciences (SPSS) program, version 16 and Microsoft Excel 2007. Data are presented as means \pm standard error (SE). The significance difference between values was estimated using one-way ANOVA and student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

In our study serum glucose level was significant high and serum insulin and estrogen levels were significant low in Ovx – diabetic group compared to control group, while in *P. Ostreatus* treated Ovx - diabetic groups there were a significant decrease in level of serum glucose and significant increase in the level of serum insulin and estrogen compared to Ovx – diabetic group (table 1).

The presented study showed that level of TC, TG and LDL-C were significantly increased while HDL-C was significantly decreased in Ovx-diabetic group when compared with control group. A significant reduction in levels of TC, TG, LDL-C while a significant elevation in level of HDL- in *P. Ostreatus* treated Ovx - diabetic groups compared to Ovx – diabetic group were observed (Table 2). Activity of PON1 and concentration of tissue NOx were significantly lower in OVX-diabetic group than control group while a significant elevation in serum PON1 activity and tissue NOx concentration in the treated groups were observed (Table 3)

ADMA levels was pronounced elevated in Ovx-diabetic group as compared with control group. While a significant reduction in ADMA level in treated Ovx - diabetic rats compared to Ovx - diabetic group was observed (Table 4).

There was a significant reduction in t-PA concentration in Ovx-diabetic group when compared to control group, which was elevated in

Table 1: Mean concentrations of fasting blood glucose, insulin and estrogen in different studied groups.

Groups	F.B.S mg/dl	Serum insulin μIU/ml	Estrogen ng/ml
Control	112.5±1.38	15.2±0.2	7.9±0.14
Control 100	120±1.29	14.9±0.12	7.7±0.2
Control 200	116.8±1.3	14.5±0.3	7.8±0.14
Sham	117±1.31	14.7±0.31	7.6±0.05
STZ-OVX	265.6±2.2 ^{a*}	8.1±0.14 ^{a*}	4±0.04 ^{a*}
STZ-OVX100	134.6±1.7 ^{b*}	11.8±0.28 ^{b*}	6.5±0.025 ^{b*}
STZ-OVX200	125.3±2 ^{b*,c*}	13.1±0.32 ^{b*,c*}	6.8±0.033 ^{b*,c#}

Significant p value < 0.05

a* = significant difference compared to control group

b* = significant difference compared to STZ-OVX group

c* = significant difference compared to STZ-OVX100 group

c# = no significant difference compared to STZ-OVX100 group

Number of animals in each group= 10

Table 2: Levels of serum lipid profile in different studied groups.

Groups	Cholesterol mg/dl	TG mg/dl	HDL -C mg/dl	LDL -C mg/dl
Control	63±1.87	80.9±3.3	58.1±1.8	74.5±0.95
Control 100	66.9±1.11	82.4±0.8	53.6±1.4	74.8±0.75
Control 200	65.7±1.84	80.6±0.02	56.2±1.38	73.6±0.66
Sham	60.3±0.85	81.9±1.1	58.3±1.1	74.5±0.84
STZ-OVX	107.5±2.17 ^{a*}	181.3±3 ^{a*}	34.9±0.5 ^{a*}	104.4±1.1 ^{a*}
STZ_OVX100	73.8±0.32 ^{b*}	92.1±0.8 ^{b*}	41.9±0.4 ^{b*}	92.9±0.69 ^{b*}
STZ-OVX200	72.2±0.92 ^{b*,c#}	86.2±0.08 ^{b*,c*}	48.5±1.4 ^{b*,c*}	77.5±0.44 ^{b*,c*}

Significant p value < 0.05

a* = significant difference compared to control group

b* = significant difference compared to STZ-OVX group

c* = significant difference compared to STZ-OVX100 group

c# = no significant difference compared to STZ-OVX100 group

Number of animals in each group= 10

Table 3: Levels of NOx and PON1 in different studied groups.

Groups	Tissue NOx μmol/g	PON 1 μmol/L
Control	26.3±0.97	219.4±2.5
Control 100	26.5±0.85	217.2±2.1
Control 200	27.5±1.13	223.2±2.2
Sham	26.1±0.38	219±2.1
STZ-OVX	13.5±0.5 ^{a*}	91.8±2 ^{a*}
STZ-OVX100	21±0.59 ^{b*}	205.4±1.1 ^{b*}
STZ-OVX200	23.8±0.74 ^{b*,c*}	213±0.9 ^{b*,c*}

Significant p value < 0.05

a* = significant difference compared to control group

b* = significant difference compared to STZ-OVX group

c* = significant difference compared to STZ-OVX100 group

Number of animals in each group= 10

Table 4: Levels of t-PA and plasma ADMA in different studied groups.

Groups	t-PA ng/ml	ADMA μmol/L
Control	3.57±0.05	0.23±0.026
Control 100	3.4±0.022	0.21±0.018
Control 200	3.6±0.12	0.22±0.043
Sham	3.5±0.06	0.38±0.046
STZ-OVX	2.8±0.028 ^{a*}	0.83±0.02 ^{a*}
STZ-OVX100	3.1±0.03 ^{b*}	0.45±0.075 ^{b*}
STZ-OVX200	3.3±0.35 ^{b*,c*}	0.41±0.097 ^{b*,c#}

Significant p value < 0.05

a* = significant difference compared to control group

b* = significant difference compared to STZ-OVX group

c* = significant difference compared to STZ-OVX100 group

c# = no significant difference compared to STZ-OVX100 group

Number of animals in each group= 10

treated Ovx - diabetic rats compared to Ovx - diabetic rats (table 4).

DISCUSSION

Endothelial dysfunction is a systemic disorder and a critical element in the pathogenesis of atherosclerotic diseases and their complications (Cibor et al, 2016). There are evidences have proved that diabetes is extremely correlated with oxidative stress, hyperglycemia and endothelial dysfunction which are believed a precursory risk factor for the evolution of cardiovascular disease such as atherosclerosis. This is consistent with the present results where serum glucose level was significantly high and a serum insulin level was significantly low in Ovx – diabetic group compared to control group. These results are in agreement with Ghaly et al. (2011) who reported the cytotoxic effect of STZ on pancreatic beta-cells causing hypoinsulinemia and hyperglycemia.

P. Ostreatus. confirms its potential effects in treated Ovx - diabetic group through significant decrease level of serum glucose and significantly increase level of serum insulin compared to Ovx – diabetic group. These results are agreed with. Jayasuriya et al. (2015) who reported that Oyster mushrooms have significant hypoglycemic effects in diabetic mice.

This may be due to rich *P. Ostreatus* with β-glycan that reduce activity glycogen synthase kinase (GSK) which is responsible for phosphorylation of glycogen synthase (GS) and reduces its activity that decrease glycogen synthesis and increase blood glucose levels (Eldar-Finkelman et al. 2010). Also it increases

insulin receptor substrate IRS-1 phosphorylation, which contributes to insulin resistance formation (Beurel et al. 2015).

Hormonal changes that accompany menopause, particularly decreased levels of estrogen hormone, have a great physiological impact that is associated with endothelial dysfunction. When the ovary which is the main organ for estrogen secretion was removed estrogen levels acutely declining (Xu-dong et al. 2012). In the present study, mean concentration of estrogen was significantly decreased in Ovx-diabetic rats as compared with control rats. *P. Ostreatus*, rich with phytoestrogens, a group of derived substances that are structurally or functionally similar to estrogen and have the ability to attach with estrogen receptors and manifested estrogen-like functions (Suman et al. 2008). This explain the significant increase in estrogen level seen in treated Ovx - diabetic rats groups compared to Ovx - diabetic rat group. These results are in regular with increased estrogen levels by natural phenolic acids in ovariectomized rats by Zych et al. 2009.

The presented study showed that level of TC, TG and LDL-C were significantly increased and HDL-C was significantly decreased in Ovx-diabetic group as compared with controls groups. These observations were found by Otamere (2011) and his core searchers that the most common lipid disorder associated with diabetes is increased level of TG,TC, low levels of HDL, and the presence of small dense as a result, more atherogenic LDL particles. This explained by Anwar et al. 2013 who observed that insulin lack

in diabetes mellitus causes a diversity of derangements in regulatory and metabolic processes. Also in menopause the loss in ovarian function is associated with alters in insulin and glucose metabolism, body fat distribution and lipoprotein profile disorders (El-Habachi et al. 2014).

Lovastatin, the hypocholesterolaemic agent present in mushrooms, may be involved in decreasing the activity of the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase enzyme, the rate-limiting enzyme for cholesterol biosynthesis. This was represented by a significant reduction in levels of TC, TG, LDL-C while HDL-C was significantly high in *P. Ostreatus* treated Ovx - diabetic groups compared to Ovx - diabetic group. Furthermore, mushroom contains pectinand β -1,3-D-glucan which are water soluble gel substances that bind to bile acids and prevent cholesterol resorption and cholesterol-bile micelle formation. These results are consistent with Alam et al. 2009, in that mushroom consumption is associated with improvements in lipid profile.

These lipid disorders in diabetes are associated with a lower serum PON1 activity and concentration in streptozotocin-induced diabetic rats Kiyıcı et al. 2010 and match the significant decrease in PON1in Ovx-diabetic group. The decrease in PON1 activity is a consequence of an alter in synthesis and/or secretion of HDL-C, or from the interaction of oxidized LDL-associated oxidized phospholipids, oxidized cholesterol ester, or lysophosphatidyl choline with the PON1's free sulfhydryl group at Cys 284 (Abdel-Wahhab et al. 2012). *P. Ostreatus*rich with phenolic compounds in addition to Naringin, vitamins E and C, folic acid and selenium possess antioxidant effects and helps in a significant elevation of PON1 in the treated groups through direct interaction with enzyme or stabilization of paraoxonase on HDL and/or its expression. (Al-Farsi and Lee, 2008).

Excessive superoxide production by the mitochondrial transport chain in hyperglycemic state (Wang et al. 2015) with increases in oxidative stress reduces NO bioavailability causing endothelial dysfunction. According to our study concentration of tissue NOx was significant lower in OVX-diabetic group than control group. This reduction is due to elevation hyperglycemia that resulting in raised activity of aldose reductase and consequently consumes NADPH that wanted as cofactor for NOS and production of NO from L-arginine (Suresh and Undurti 2006). Also Chew and Watts 2004 reported that oxidative stress resulting in oxidation BH4, a co-factor that

modulates NO generation causing uncoupling of eNOS and decreased NO generation.

Decreased NO production is associated with increased levels of ADMA, an endogenous suppressor of eNOS during competition with L-arginine generation (Kirkby et al. 2016). This elevation in ADMA levels was pronounced found in Ovx-diabetic group as compared with control group. This may be explained that ADMA elevation in diabetes is due to impairment of dimethylarginine dimethylaminohydrolase (DDAH) action result from increase in glucose, oxidative stress and oxLDL resulting in increased the activity of S-adenosylmethionine-dependentmethyl transferases, which lead to increased ADMA synthesis (Mudau et al. 2012).

As Oyster mushroom is rich in protein, carbohydrates and vitamins. Also it contains antioxidants such as flavonoids, gallic acid,, lycopene, chlorogenic acid and β -carotien (Reis et al. 2011) besides being significantly involved in human detoxification processes (Alam et al. 2011).

Crude polysaccharide of *P. Ostreatus* is a potent activator of NOS (Mitra et al. 2013) and in homogenous with our results with increased NO in treated Ovx - diabetic rats compared to Ovx - diabetic rat. As well as lovastatin enhanced NO bioavailability and upregulation of eNOS via inhibition in the mevalonate pathway (Ishida et al. 2012) which impairs NO bioavailability through inhibition of eNOS mRNA stability and eNOS protein phosphorylation at Ser1177. Additionally, *P. Ostreatus*rich with niacin has been demonstrated to decrease plasma ADMA by reducing synthesis of methyl arginines because the metabolism of niacin requires large amounts of methyl groups. Consequently, a methyl donor, S-adenosylmethionine(SAM), could be depleted and become unavailable for the methylation of proteins. This hypothesis is consistent with the simultaneous decrease in ADMA observed by Westphal et al. 2006 and regular with a significant reduction in ADMA level in treated Ovx - diabetic rats compared to Ovx - diabetic group.

In the same manner, excessive intracellular glucose with increasing oxidative stress result not only in depletion of NADPH but also increase (PAI-1) gene expression (Paneni et al. 2013). PAI-1 has an inhibitory activity against t-PA, binds rapidly to t-PA and forms an inactive t-PA-PAI-1 complex (Kohler and Grant. 2000). This elucidate the significant reduction in t-PA concentration in Ovx-diabetic group when compared to control group, which return to a reduction in endothelial

cells capacity to secrete t-PA in response to a fibrinolytic stimulus (Umpaichitra et al. 2005). This reduction is improved by Lovastatin, a mushroom content, which increases tissue type t-PA and decreases PAI-1 production on experimental created peritoneal adhesion model in rats (Lalountas et al. 2010). This elevation is obviously found in treated Ovx - diabetic rats compared to Ovx - diabetic rat group. The effects of statins on the fibrinolytic pathway are mainly mediated by the increased t-PA expression and activity (Aarons et al. 2007).

CONCLUSION

Pleurotus ostreatus is an edible mushroom with high nutritional and biomedical importance in improving biochemical changes of endothelial dysfunction, initial event in progress of atherosclerosis, since it contains a number of bioactive components. Due to its high nutritional values, *p. ostreatus* has great health advantages through the adjustment of physiological functions such as a strong hypoglycemic effect, beneficial effects on lipid profile, improving endothelium-dependent vasodilatation, decreasing intravascular thrombus propagation. Moreover, it contains Phytoestrogens that exert potent estrogen-like effects which showed a significant increase in estrogen. Therefore, it can be used widely to overcome the possible side effects of HRT symptoms associated with menopause and in improving biochemical changes of endothelial dysfunction initial event in progress of atherosclerosis.

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