



## Original article

## Effect of $\gamma$ -oryzanol on hyperlipidemia and thrombus formation in mice treated with poloxamer-407

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## ABSTRACT

**Introduction:** Hyperlipidemic and thrombotic events are the most common risk factors for death related to cardiovascular diseases.  $\gamma$ -Oryzanol (OZ) is the active chemical constituent of *Oryza sativa* bran oil. It is reported for its antihyperlipidemic activity.

**Aim:** Our study was aimed to evaluate effect of OZ on atherothrombotic events, i.e. hyperlipidemia and thrombosis in animal model.

**Material and methods:** Swiss albino male mice were divided into four groups. Animals were treated with atorvastatin (2 mg/kg, p.o., as the standard drug) and OZ (100 mg/kg, p.o.) up to 3 days. On the 3rd day poloxamer-407 (500 mg/kg, i.p.) was administered to induce hyperlipidemia. Thrombotic plaque was induced using  $\text{FeCl}_3$  (50%). Animals were sacrificed after 24 h from induction of hyperlipidemia. Blood was collected for estimation of lipid profile, coronary disease risk factors, blood coagulation parameters i.e. APTT and PT. Liver was isolated for estimation of oxidative stress parameters. Further, the effect of therapy on thrombus formation was observed by histopathology of carotid artery.

**Results and discussion:** Treatment with OZ was found to improve the serum lipid profile, reduce coronary risk factors, and to decrease oxidative stress. Thrombus formation was found to be reduced on histopathological examination. OZ prolonged APTT and PT.

**Conclusions:** OZ found to be therapeutically efficient in hyperlipidemic and atherosclerotic risk management in the animals which may be due to its anti-oxidative stress activity.

## 1. INTRODUCTION

Hyperlipidemia has been considered as a predominant contributing factor for atherosclerosis and resultant adverse cardiovascular events.<sup>1</sup> Hyperlipidemia can be designated as elevated cholesterol, and lipoproteins i.e. low density lipoprotein (LDL) along with low or unaltered high density lipoprotein (HDL) levels in blood.<sup>2</sup> Intraperitoneal administration of poloxamer-407 has been found to induce hypertriglyceridemia within 24 h. It deviates normal lipoprotein level from its regular range by lipoprotein lipase inhibition and HMG-CoA reductase up regulation, the enzyme for regulation of cholesterol synthesis pathway.<sup>3</sup> Previous studies reported that poloxamer-407 elevates plasma cholesterol and triglycerides following intraperitoneal injection.<sup>4</sup> It is apparent that hyperlipidemia is responsible for thrombosis, follows atherosclerotic vascular damage.<sup>5</sup> The atherosclerotic plaque formed in pathogenesis cascade, leads to inhibition of visceral hemoperfusion and increased risk of cardiovascular death and it gets ruptured after some time. It subsequently damages endothelium followed by activation of platelets and coagulation factors which are liable for thrombus formation.<sup>6</sup> Atrovastatin, a member of statins, inhibits HMG-CoA reductase in competitive manner,<sup>7</sup> reduces cholesterol biosynthesis, upregulates LDL receptor and promotes uptake of LDL. Further it is reported to upregulate apolipoprotein A1 (Apo A1) synthesis and HDL levels by unknown mechanism.<sup>8</sup> Augmented risk of hyperglycemia, cataract, erectile dysfunction and neurodegeneration have been reported by statin treatment.<sup>9</sup> Since rhabdomyolysis is critical muscular problem found to occur after atorvastatin therapy,<sup>8</sup> requisite of substitute remedy has been emerged to minimize such clinical event so that the global burden of cardiovascular risk as well as drug related side effects could be abridged.

In current scenario the natural plant based nutritional supplements are emphasized to rid the patients from several diseases. Hence plant based nutritional and herbal by-products are center of interest for investigation. Rice bran oil contains various bioactive compounds with nutritional values viz. polyphenols and their derivatives, ferulic acids and their sterol esters i.e. cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, campesteryl ferulate. Further it is enriched with vitamin C, tocopherols, inositols and phytidic acid.<sup>10,11</sup>  $\gamma$ -Oryzanol (OZ), a ferulic esters' combination, ameliorates lipid profile by regulating lipoproteins and total cholesterol levels. Further it has been reported for its atherosclerotic index reduction activity as well as anti-lipid peroxidation profile by virtue of its antioxidant activity.<sup>12</sup>

## 2. AIM

The study was designed to evaluate potential of OZ for treatment of hyperlipidemia and thrombotic events in animals treated with poloxamer-407 and FeCl<sub>3</sub>.

## 3. MATERIAL AND METHODS

### 3.1. Chemicals

OZ was procured from TCI chemicals, India. Poloxamer-407 (Pluronic RF-127) was obtained from Sigma-Aldrich, USA. Atorvastatin was gifted by Troikaa Pharmaceuticals, India. Diagnostic kits for lipid profile were purchased from Lab Care Diagnostics, India. Diagnostic kits for APTT and PT were purchased from Diagnostica Stago, France.

### 3.2. Animals grouping and treatment

After approval of protocol for study by Institutional Animal Ethics Committee (protocol no. IP/PCEU/MPH/14-1/010); Swiss albino male mice (60–40 gm) were used for the study. Mice were divided in different groups (each group  $n = 6$ ) as: normal control (NC), disease induced (DI), standard treated (ST) and OZ suspension treated (OZS). NC and DI animals were treated with 0.5% CMC for 3 days. In DI animals 30% w/v poloxamer-407 (500 mg/kg, i.p) was administered on 3rd day to induce hyperlipidemia. In ST and OZS animals atorvastatin (2 mg/kg per day, p.o.) and OZ (100 mg/kg per day, p.o.) were administered respectively for 3 days before administration of poloxamer-407 to induce hyperlipidemia. After 24 h of poloxamer-407 injection; blood and liver samples were collected from animals to estimate various biochemical parameters.

### 3.3. Determination of body weight

Change in body weight was measured before and after therapy. The percentage change in body weight was calculated.

### 3.4. Estimation of lipid profile and coronary risk predictor indices

Total cholesterol (TC), triglycerides (TG) and HDL levels were estimated in serum by procedure recommended by commercially available diagnostic kits (Lab Care Diagnostics, India). Serum VLDL and LDL were determined by using Friedewald formula<sup>13</sup>:

$$\begin{aligned} \text{VLDL} &= \text{TG}/5, \\ \text{LDL} &= \text{TC} - \text{HDL} - \text{VLDL}. \end{aligned}$$

Next, coronary risk predictor indices i.e. atherogenic index (AI)<sup>14</sup> and LDL to HDL ratio<sup>15</sup> were measured.

$$\text{AI} = (\text{TC} - \text{HDL}) / \text{HDL}.$$

### 3.5. Determination of blood coagulation parameters

APTT and PT were measured by use of commercially available diagnostic kits from C.K. PREST, Diagnostica Atago, France.

### 3.6. Oxidative stress parameters

Animals were euthanized, livers were removed and washed by ice cold buffer. Tissues were minced and homogenized in buffer with pH 7.4 using 25 stroke of homogenizer (Remi Motors) at the speed of 2500 rpm. Clear superna-

tant was taken to estimate catalase activity (CAT),<sup>16</sup> superoxide dismutase activity (SOD)<sup>17</sup> and reduced glutathione level (GSH)<sup>18</sup> while tissue homogenate was used to estimate malondialdehyde (MDA)<sup>19</sup> levels. All parameters were measured as per previously described procedure.

### 3.7. Formation of thrombosis

In diazepam (100 mg/kg) and ketamine (80 mg/kg) anaesthetized mice; after exposure of carotid artery; 50% FeCl<sub>3</sub> soaked whatmann paper piece (2 × 1 mm) was applied topically for 10 minutes to produce thrombosis. After 60 minutes of the paper application carotid artery was dissected out and embedded in paraffin<sup>20</sup> and 5 μm thick sections were taken after paraffinization of the artery. Hematoxylin eosin was used for staining of the sections.<sup>21</sup>

### 3.9. Statistical analysis

The statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test using Graph pad prism 5.03.

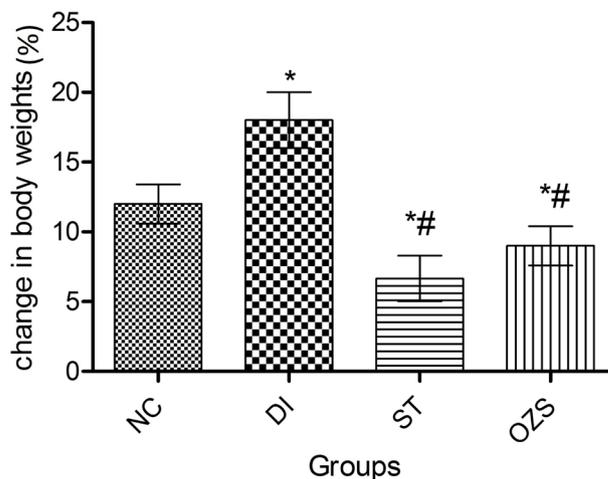
## 4. RESULTS

### 4.1. Body weight

After 3 days of treatment period, DI animals had exhibited significant weight gain as compared to NC animals. ST and OZS groups showed significantly reduced body weight compared to DI animals (Figure 1).

### 4.2. Lipid profile and coronary risk predictor indices

After treatment with poloxamer-407, TC, TG, LDL, VLDL levels were elevated significantly in DI animals as compared to the NC group. Significant increase in HDL level was observed in the DI group. Significant reduction in TG, LDL, TC and VLDL levels was observed in the ST and OZS



**Figure 1. Effects of OZ treatment on weight. Comments: All values are expressed as mean ± SEM; n = 6, \* P < 0.05 vs. NC, # P < 0.05 vs. DI.**

group animals. Moreover, significant increase in HDL level was observed in the ST and OZS animals. Similarly LDL level was improved significantly in OZS treated animals when compared with the ST group. The AI value, LDL and HDL ratio were significantly increased in the DI animals. However, all these values remained to be normal in the ST and OZS groups (Table 1).

### 4.3. Blood coagulation parameters

Statistically insignificant reduction were observed in APTT as well as PT values in the DI group compared to the NC group. Pretreatment with OZ and atorvastatin prolonged APTT and PT time as compared to the DI group (Table 2).

### 4.4. Oxidative stress parameters

After administration of poloxamer-407 significantly increased MDA levels, reduced SOD, CAT activity and GSH levels were observed in the DI group animals when com-

**Table 1. Coronary risk factors indices.**

Groups	NC	DI	ST	OZS
TC, mg/dL	102.41 ± 4.46	875 ± 5.41*	367.50 ± 8.90*#	351.417 ± 3.36*#
TG, mg/dL	63.33 ± 5.09	946.31 ± 3.26*	251.82 ± 7.47*#	469.78 ± 5.21*#
VLDL, mg/dL	12.66 ± 1.01	189.26 ± 0.65*	50.36 ± 1.4*#	93.95 ± 1.04*#
LDL, mg/dL	21.56 ± 3.89	589.91 ± 11.91*	184.76 ± 9.37*#	119.62 ± 8.11*#
HDL, mg/dL	71.18 ± 5.54	32.18 ± 2.14*	132.36 ± 6.9*#	137.83 ± 6.40*#
AI	0.5 ± 0.09	9.43 ± 0.97*	1.80 ± 0.13*#	1.58 ± 0.14*#
LDL and HDL ratio	0.36 ± 0.09	7.18 ± 0.78*	1.42 ± 0.12*#	0.89 ± 0.11*#

Comments: All values are expressed as mean ± SEM; n = 6, \* P < 0.05 vs NC, # P < 0.05 vs DI.

**Table 2. Coagulation parameter results.**

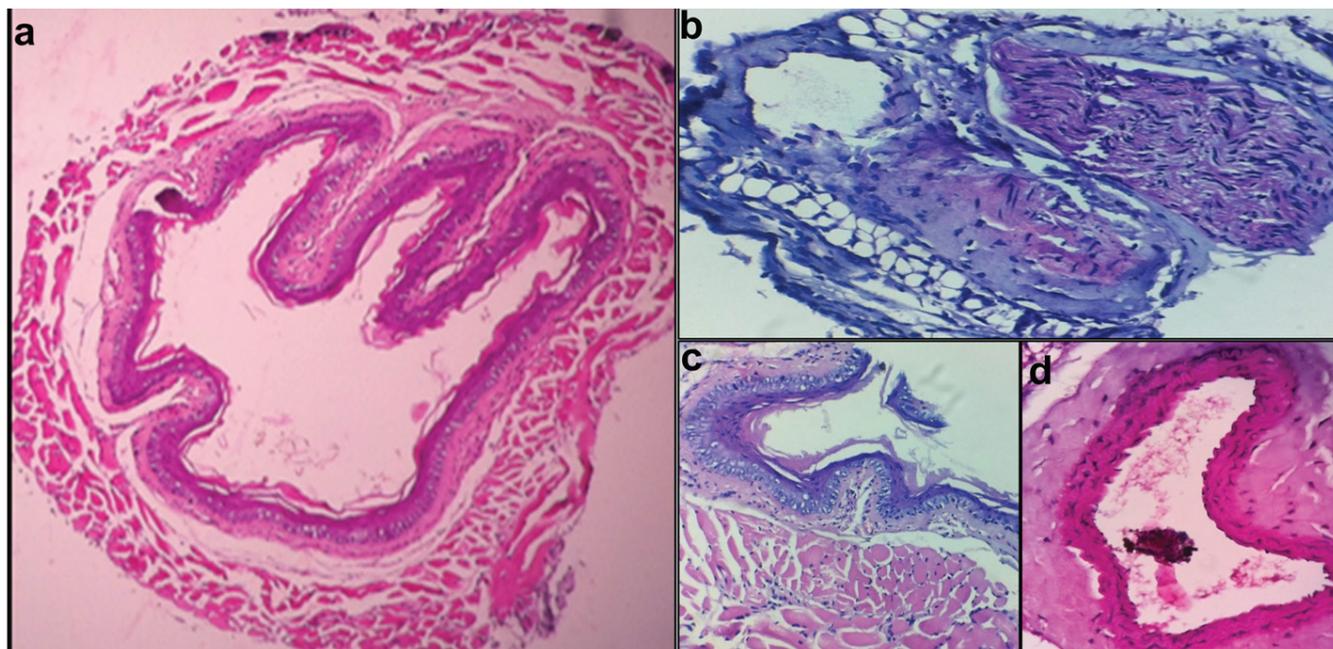
Groups	NC	DI	ST	OZS
APTT (s)	20.33 ± 0.92	18.65 ± 0.37	21.98 ± 2.77#	21.08 ± 0.73#
PT (s)	11.83 ± 0.48	10.83 ± 0.60	12.50 ± 1.76	12.17 ± 0.79

Comments: All values are expressed as mean ± SEM; n = 6. # P < 0.05 vs DI.

**Table 3. Changes in oxidative stress profile.**

Groups	NC	DI	ST	OZS
MDA levels, nmole/gm protein	0.53 ± 0.06	2.98 ± 0.65*	0.65 ± 0.09 <sup>#</sup>	0.36 ± 0.07 <sup>#</sup>
SOD activity, U/min/gm protein	4.00 ± 0.44	1.44 ± 0.29*	4.39 ± 0.29 <sup>#</sup>	2.74 ± 0.49 <sup>#</sup>
CAT activity, U/min/gm protein	0.03 ± 0.00	0.02 ± 0.00*	0.05 ± 0.00 <sup>#</sup>	0.04 ± 0.00 <sup>#</sup>
GSH levels, µg/ mg protein	6.09 ± 0.20	4.25 ± 0.06*	6.31 ± 0.23 <sup>#</sup>	5.80 ± 0.16 <sup>#</sup>

Comments: All values are expressed as mean ± SEM; *n* = 6. \**P* < 0.05 vs. NC, <sup>#</sup> *P* < 0.05 vs. DI.



**Figure 2. Hematoxylin-eosin stained cross section of carotid arteries: NC group (A), DI group (B), ST group (C), OZS group (D).**

pared with the NC group. Significantly reduced MDA levels, increased SOD, CAT activity and GSH levels were observed in the ST and OZS groups when compared with the DI group (Table 3).

#### 4.5. Histopathology

Histopathological study showed thrombus formation and atherogenicity in poloxamer-407 administered (DI) animals. Significant damage in endothelium cells of all carotids arteries was observed. In poloxamer-407 treated animal severe destruction of central lipid layer and endothelial cells was observed. However in the ST and OZS groups significant reduction in damage to endothelium and lipid core were observed as shown in Figure 2.

## 5. DISCUSSION

Atherosclerosis and hyperlipidemia have been found as major factor for cerebrovascular diseases, coronary artery diseases and peripheral vascular diseases.<sup>22,23</sup>

Poloxamer-407 treated DI animals exhibited increased body weight. OZS animals exhibited comparatively normal body weight. Increased serum TC, TG and LDL levels increases risk of coronary heart disease.<sup>24,25</sup> Poloxamer-407

increases TC, TG, LDL, VLDL levels.<sup>26</sup> In the present investigation, DI animals showed significantly elevated TC, TG, VLDL and LDL levels by up regulation of HMG-CoA reductase enzyme 3 and decreased HDL levels. In previous studies OZ had been found to reduce TC, TG, VLDL as well as LDL and elevate HDL levels.<sup>11</sup> In current study OZS animals exhibited improvement in lipid levels by decreasing TC, TG, LDL as well as VLDL levels and elevating HDL level. Poloxamer-407 has been reported to increase AI, which is a marker of atherosclerosis.<sup>27</sup> Our study also reports raised value of AI in DI animals which was found to be lower in OZS animals as compared to DI animals. LDL and HDL ratio is a marker of vascular risk which should be low for preventing the coronary disease risks. DI animals exhibited high LDL and HDL ratio and were found to be atherogenic. However, all these values returned to the normal levels by OZ treatment. Hypercholesterolemia is convicted to be the risk factor for accumulation of oxidized LDL (oxi-LDL) which accelerate foam cells formation and cascade of several atherosclerotic events.<sup>28,29</sup> Oxidative stress occurs when antioxidant/prooxidant homeostasis is imbalanced and reactive oxygen species (ROS) production is elevated.<sup>30</sup> Lipid peroxidation leads to the production of MDA as a by-product<sup>31</sup> which is detrimental to body. Poloxamer-407 has been reported to aggravate oxidative damage and formation

of ox-LDL.<sup>32</sup> Antioxidant enzymes inhibit the lipoprotein oxidation which in turn provides a supportive mechanism for defense in the body against ROS and decreases the risk of atherogenesis. In our study because of elevated cholesterol and LDL levels in the poloxamer-407 induced atherosclerotic animals MDA levels were found to be upraised with reduction of anti-oxidative enzymes i.e. SOD, CAT and GSH. Moreover, OZ significantly inhibited lipid peroxidation. It markedly improved the antioxidant enzymes such as CAT and GSH levels which collectively indicated a protective and preventive effects of OZ in oxidative stress induced atherosclerotic complications. No significant changes in SOD activities were observed in our study. Oxidative stress in metabolic syndromes are associated with thrombotic events in the body.<sup>33</sup> Studies reported the hyperlipidemia and hypercoagulability markers such as APTT and PT are associated with each other.<sup>34</sup> In previous studies, it is reported that on endothelial cells, accumulation of spherical bodies filled with ferric ion reacts to adhere as well as support formation of aggregates. So FeCl<sub>3</sub> can promote this effect in carotid artery. It can also affect the function of adhesion proteins like fibrinogen, collagen, von Willebrand factors and thereby promote platelet adhesion.<sup>35,36</sup> In our study APTT and PT were reduced in the DI animal group in statistically insignificant manner which were maintained to the normal by pre-treatment with OZ which indicates positive effect on both intrinsic as well as extrinsic coagulation process.

Histological study of carotid artery, showed reduced damage of endothelial and lipid layers with OZ treatment which indicated beneficial effects of OZ against thrombotic events. Hence OZ improves serum lipid profiles, reduces coronary risk factors, decreases the oxidative stress in the body, affect coagulation pathways, and decreases the risk of thrombus formation in atherosclerotic/ hyperlipidemic mice.

## 6. CONCLUSIONS

OZ has beneficial effects on hyperlipidemia and atherosclerotic events mainly by improvement of lipid profile, inhibition of thrombotic events and anti-oxidative stress effects.

### Conflict of interest

There is no conflict of interest for this paper.

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