Evaluation of the hypolipidemic activity of 6,7-dimethoxycoumarin on placental tissue factor mRNA expression in experimental anti-phospholipid syndrome

Annamalai Amarnath, Veeraval Lenin, Govindaraju Archunan

Department of Animal Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

ABSTRACT

Background: Anti-phospholipid syndrome is a thrombogenic and systemic autoimmune disorder that influences fetal life throughout gestation period. Over expression of tissue factor on the surface of monocyte(s) is reported to be a major causative agent in inducing anti-phospholipid antibody-mediated placental thrombosis and fetal loss in pregnant women. The over expression of tissue factor is proposed to be due to high levels of blood cholesterol and oxidized lipoproteins. **Objective:** In this study, we report the lipid-lowering property and anti-tissue factor activity of one of the naturally occurring coumarin derivates 6,7-dimethoxycoumarin, found aplenty in Chinese medicinal plant *Artemisia scoparia*, and its effect on tissue factor mRNA expression in experimental anti-phospholipid syndrome. **Materials and Methods:** Adult female mice were immunized with cardiolipin and beta-2-glycoprotein-1 to induce experimental anti-phospholipid syndrome. Female mice with high titer of aCL were allowed to mate with male, and the female mice were treated with 6,7-dimethoxycoumarin on a daily dose of 5 mg/kg body weight from day 3 to day 15 of gestation. On day 18 of pregnancy, all the animals were dissected to measure biochemical parameters in blood, and TF mRNA expression levels were measured in placenta. **Results:** Treatment with 6,7-dimethoxycoumarin significantly reduced the levels of cholesterol and plasma lipids by its potent hypolipidemic property, which eventually reduced the over-expression tissue factor at mRNA levels in placenta. We believe that further studies in animal model would reveal the potential therapeutic properties of 6,7-dimethoxycoumarin against anti-phospholipid syndrome. **Conclusion:** The 6,7-dimethoxycoumarin is capable to reduce the expression of TF in placenta at the mRNA level and thrombus generation indirectly by its potent anti-TF and anti-oxidant activities.

Keywords: 6,7-Dimethoxycoumarin, anti-phospholipid syndrome, fetal loss, thrombosis, tissue factor

INTRODUCTION

Anti-phospholipid syndrome (APS), a thrombogenic disorder, plays a decisive role in women of reproductive age groups, more particularly, production of antibodies to the cell membrane phospholipids target's placenta where they induce thrombosis and devastate growing fetuses during pregnancy. Reports from the past few decades brought out several crucial, independent factors involving both directly and indirectly, in inducing APS-mediated placental thrombosis. They are abnormal expression of tissue factor (TF) on monocytes, deposition of complement proteins in the placental beds,[1] displacement of Annexin A5 from non-cellular phospholipids,[2] and several cell-mediated events.[3] Tissue factor, an essential molecule for uterine homeostasis during gestation[4] and to initiate thrombosis under pathological condition,[5] is a 47 kDa trans-membrane protein that plays a central role in anti-phospholipid antibody-mediated placental thrombotic manifestations.

Numerous *in vitro* and *in vivo* studies support the precise role of anti-phospholipid antibodies (aPL) in mediating TF over expression on monocytes, and pro-inflammatory responses that lead to fetal death. In addition to the above

Address for correspondence:
Dr. Govindaraju Archunan, Department of Animal Science,
Bharathidasan University, Tiruchirappalli, Tamil Nadu - 620 024,
India. E-mail: archunan@bdu.ac.in

factors, cholesterol and oxidized lipoproteins (ox-LDL) are reported to play an indispensable role in augmenting TF expression and its functional activity.[6] In parallel, aPL antibodies have been reported to cross react with ox-LDL in patients with APS that results in development of atherosclerosis.[7] In order to reduce the risk of inflammation, blood coagulation, and fetal loss in aPL antibody-induced mice, statins, also known as cholesterol-lowering agents, have been documented to diminish the expression of tissue factor and protease-activated receptors 2 (PAR2).[8] A recent research on statin's ability to lessen thrombosis and fetal loss in patients with APS revealed that they possess the property of affecting gene expression and cellular functions of immune cells.[9] The derivatives of several novel coumarin have also been identified as lipid-lowering agents,[10,11] among them, 6,7-dimethoxycoumarin has been reported to lower plasma lipid and lipoprotein cholesterol levels.[12] We found that 6,7-dimethoxycoumarin reduce the risk of TF-dependent aCL-mediated placental thrombosis in the mice model (unpublished data). In the present study, we demonstrate the effectiveness of 6,7-dimethoxycoumarin on lowering plasma lipid and lipoprotein cholesterol levels that eventually reduces expression of TF-mRNA in the placenta of mice having experimental anti-phospholipid syndrome.

MATERIALS AND METHODS

Materials
6,7-dimethoxycoumarin and cardiolipin (> 99% purity) were procured from Sigma Aldrich (St. Louise, USA). Freund's complete and incomplete adjuvants were procured from Bangalore Genie, India. One-step RT-PCR kit was purchased from Genet-bio, Chungcheongnam-do, South Korea (Cat. No. R-4000).

Experimental design
Female Balb/c mice (Bioneeds, Bangalore), 8 to 10 weeks old, weighing 20 - 30 gm were used throughout the study. Mice were acclimatized to laboratory conditions with 12 hr dark/12 hr light period with temperature ranging from 25 ± 2 °C and were given *ad libitum* access to a balanced diet. All the experiments were carried out as per the approval of institutional animal ethical committee, Bharathidasan University, Tiruchirapalli.

60 healthy adult mice were initially taken into the study and were divided into 2 groups as group I (n = 40) and group II (n = 20). Anti-phospholipid antibody syndrome was induced in group I mice by intramuscularly injecting 25 µl of cardiolipin (CL) and 25 µg beta-2-glycoprotein I (β2GPI) emulsified with Freund's complete adjuvant. It was followed by 3 successive booster doses with Freund's

incomplete adjuvant at 2-week intervals. Three weeks after the final booster dose, all the mice from both groups I and II were bled via tail vein, serum was collected, and aCL antibody titer was measured as published previously. Mice (from group I) that possessed significantly higher levels of aCL titer were subjected to further experimentation, and remaining mice were discarded. Animals with higher levels of aCL (n = 21) were randomly divided into 3 groups as group Ia, Ib, and Ic each with 7 animals. Cardiolipin-untreated animals (n = 14) were equally separated into 2 groups as group IIa and IIb. All the experimental animals were allowed to mate with proven stud male, and female mice were continuously monitored for every 4 hours to observe vaginal plug formation. The day we examined vaginal plug was assumed as day 1 of pregnancy. From day 3 of pregnancy, group Ia and group IIa animals were intraperitoneally injected with 6,7-dimethoxycoumarin (5 mg/kg body weight), and group Ib animals were subcutaneously injected with heparin until day 15 of pregnancy. Group Ic and group IIb animals were left as a positive control for APS and untreated control, respectively. Figure 1 describes in detail of the experimental groups.

### Measurement of biochemical parameters
On the 18th day of pregnancy, all the animals were bled via tail vein, serum was separated and stored at 4 °C, and then the mice were euthanized by cervical decapitation. Placenta was rapidly collected from both experimental and control animals, washed in ice-cold PBS and stored at -80 °C for further experimentation. Biochemical parameters such as urea, total protein, glucose, creatinine, bilirubin, albumin, cholesterol, and plasma enzymes such as AST, ALT, ALP, and LDH were measured in serum using Auto analyzer with commercial kits purchased from Bio Systems S.A., Barcelona, Spain.

Statistical analysis
All results were analyzed using SPSS software version 11.5, and values are given as mean ± standard deviations unless otherwise noted. Student paired 't' test and one way ANOVA were used to compare the means of different groups, and the significance was noted by '*P*' value and, a '*P*' value less than 0.05 was considered to be significant.

RNA isolation
Total RNA from the placenta of all the experimental groups were isolated by Trizol method. Briefly, the placenta was homogenized with 1 ml of TRIzol reagent and incubated at room temperature for 5 to 15 min. The homogenates were then centrifuged at 12,000 RPM for 10 min at 4°C, and the resultant supernatant was transferred into fresh tubes. 200 µl of chloroform was added into each of the tubes containing the supernatant and shaken well for 15 seconds, and then incubated at
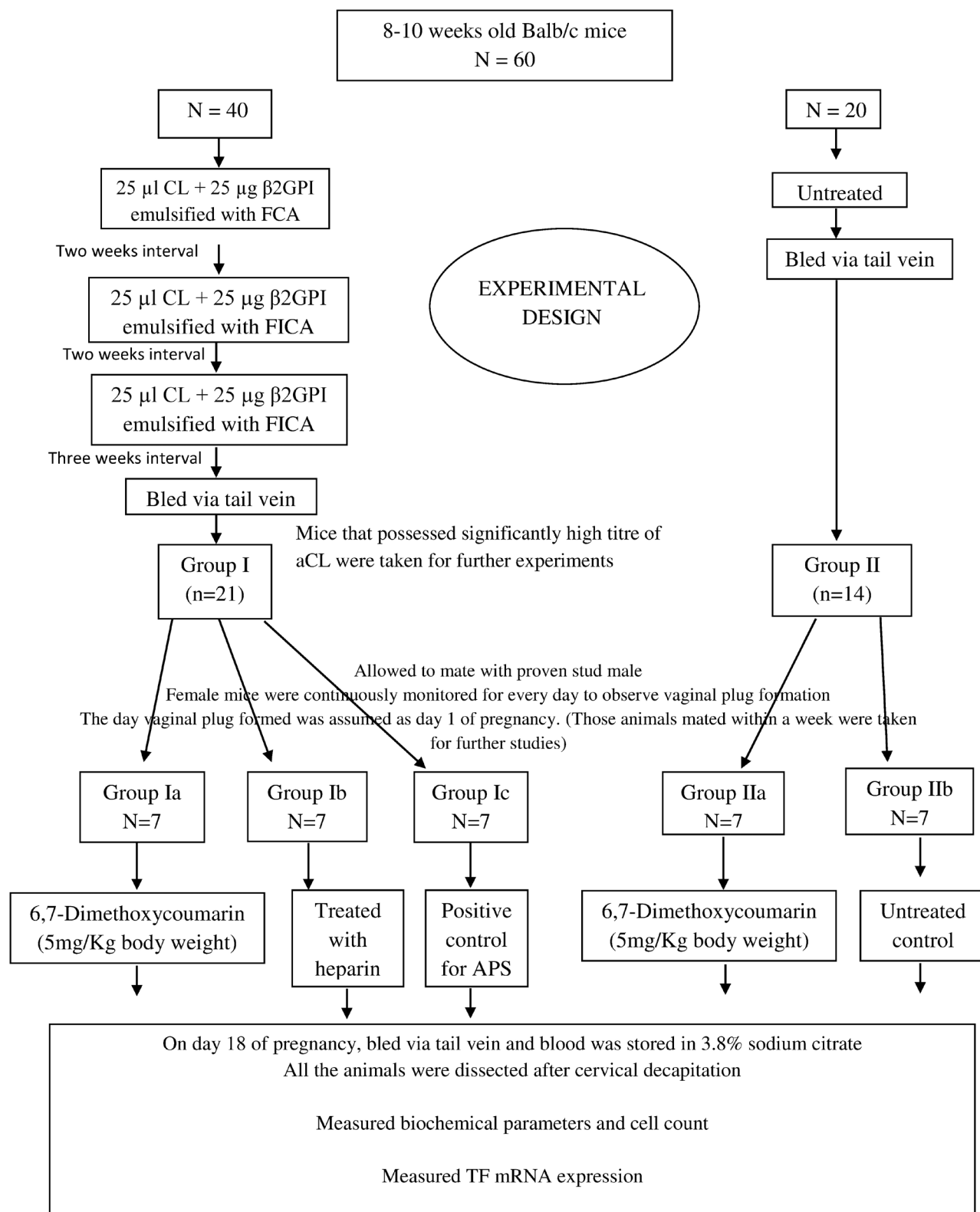
Figure 1: Details of experimental design in the present study

room temperature for 15 min. They were then centrifuged at 12,000 RPM for 15 min, and again transferred the aqueous phase into fresh tubes in which 500 µl of iso-propanol was added and vortexed for 10 seconds and incubated at room temperature for 10 min. The tubes were then centrifuged at 12,000 RPM for 8 min at 4 °C, and

the resultant pellets were collected. 1 ml of 75% ethanol was added into the pellet and centrifuged at 7500 RPM for 5 min at 4 °C. The tubes were then air-dried to remove the ethanol and added nuclease-free water. The concentration of total RNA was determined spectrophotometrically at the absorbance of 260/280 nm.

RT-PCR analysis

The relative expression patterns of TF-mRNA in all the experimental groups were measured and normalized with housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using one-step RT-PCR kits from GENET BIO (Cat. No. R-4000) according to the manufacturer's instruction. The following primers were used:
Tissue Factor
Sense, 5'-TGCTTCTCGACCACAGACAC-3';
Antisense 5'-TAAAAACTTTGGGGCGTTTG-3'.

GAPDH
Sense 5'-CCC ACT AAC ATC AAA TGG GG-3'
Antisense 5'-CCT TCC ACA ATG CCA AAG TT-3'

5 μg of total RNA were taken for the synthesis of cDNA, which were further amplified by PCR. The RT inactivation was performed at 95 °C. Totally, 30 cycles of amplification were performed as follows: i) denaturation at 95 °C for 45 sec, ii) annealing at 60 °C for 40 sec, and iii) extension at 72 °C for 40 sec. Final extension were carried out at 72 °C for 5 min. All the PCR products were loaded in 2% agarose gel with 100 bp molecular weight marker for comparison.

RESULTS

The level of anti-cardiolipin antibody in the serum of group I mice was found to be significantly high (titer value = 20480) when compared to group II mice (titer value = 680) [Figure 2]. Table 1 exemplifies the effect of 6,7-dimethoxycoumarin on hematological parameters of experimental and control groups. All the biochemical parameters did not show any significant changes among the different experimental and control groups, except lipid profiles. Lipid profiles such as cholesterol, TG, and LDL levels were found to be high in aCL-induced mice (group Ic), whereas there was a significant reduction ($P < 0.01$) upon treatment with 6,7-dimethoxycoumarin (group Ia). In contrast, there were no changes in HDL among the different group of animals and a moderate increase in VLDL levels in group Ic mice ($P < 0.05$). Likewise, a reflective increase in the liver enzymes such as LDH, ALT, AST, and ALP were seen in aCL-induced animals (group Ic) when compared to untreated control animals (group IIb) [Table 2]. On treatment with 6,7-dimethoxycoumarin, a significant reduction of these enzymes was noticed, which was not significant when compared to group IIb. Table 3 reveals the variation in cell counts among different experimental and control groups. RBC and WBC seemed to be not significantly varied between the control and experimental groups. In contrast to that, the platelet levels were low in group Ic mice and slightly increased in group Ia mice, which were moderately significant ($P < 0.05$), upon treatment with 6,7-dimethoxycoumarin.
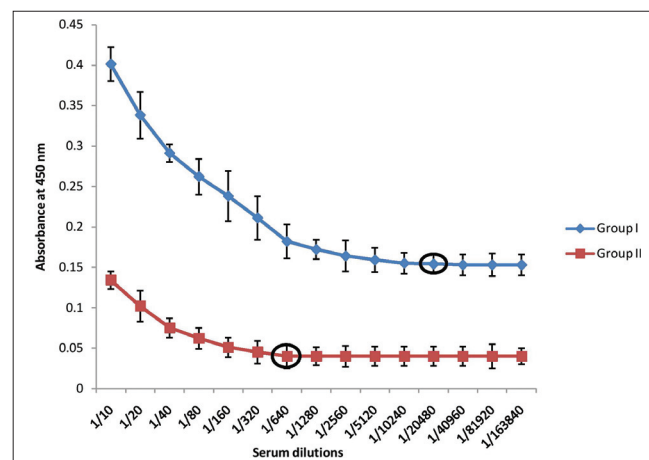
Figure 2: aCL titer was found to be higher i.e. 20480 in experimental CL immunized experimental mice than that of control group i.e. 640. Black circle indicates the saturation points for aCL. Data represents mean absorbance ± standard deviation

Table 1: Hematological parameters of all the experimental and control groups

Parameter	Urea	Protein Total	Glucose	Creatinine	Bilirubin	Albumin	Cholesterol	TG	VLDL	LDL	HDL
Units	mg/dl	g/dl	mg/dl	mg/dl	mg/dl	g/dl	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl
Group Ia	18.4 ± 1.7	4.4 ± 0.4	113 ± 12.3	0.7 ± 0.1	0.3 ± 0.1	1.9 ± 0.3	132.9 ± 12.3	78.1 ± 1.4	14.8 ± 0.9	27.4 ± 0.9	33.3 ± 0.3
Group Ib	18.4 ± 2.1	4.8 ± 0.5	109.0 ± 4.1	0.5 ± 0.0	0.4 ± 0.1	1.9 ± 0.2	134 ± 8.2	78.4 ± 1.1	15 ± 0.3	27.8 ± 1.0	31.0 ± 0.1
Group Ic	18.1 ± 1.6	4.4 ± 0.4	112.9 ± 12.1	0.5 ± 0.0	0.4 ± 0.1	1.7 ± 0.2	177.6 ± 8.6*	92.6 ± 3.5*	18.8 ± 0.5#	38.1 ± 0.8*	31.4 ± 1.0
Group IIa	18.0 ± 1.3	4.7 ± 0.4	106.1 ± 7.3	0.6 ± 0.1	0.4 ± 0.1	2.0 ± 0.1	133.0 ± 10	77.9 ± 1.7	14.9 ± 0.6	29.0 ± 0.6	31.7 ± 0.8
Group IIb	18.7 ± 1.8	4.9 ± 0.5	109.1 ± 5.5	0.6 ± 0.1	0.4 ± 0.1	2.0 ± 0.1	132.7±9.2	75.2 ± 1.9	14.6 ± 0.2	28.5 ± 0.5	30.8 ± 0.5

*$P < 0.01$, #$P < 0.05$, Cholesterol, Triglyceride, VLDL, and LDL levels were significantly high in positive control mice for APS. Lipid lowering property of 6,7-Dimethoxycoumarin significantly reduced the lipid profile levels in group Ia mice. There is no significant variation in all other biochemical parameters between the experimental and control groups

Expression of TF mRNA and co-amplified reference gene GAPDH in experimental and control groups [Figure 3]. The level of TF mRNA was observed high in aCL-induced positive control mice population (group Ic); however, treatment with 6,7-dimethoxycoumarin significantly reduced TF mRNA in group Ia mice comparable to that seen in control groups (groups IIa and IIb). Figure 4 illustrates the normalized ratios of TF mRNA and GAPDH in all the experimental and control groups; the results were obtained by densitometry analysis.

DISCUSSION

There are multiple mechanisms pertaining to the thrombosis occur in patients with APS, but the most crucial one that influence the generation of thrombus is the up-regulation of TF pathway in neutrophils by aPL antibodies.[13] Experimental evidence suggested that the procoagulant activity of TF and TF mRNA levels are increased in patients with APS;[14] further, the PAR2-dependent signaling mechanism was also observed, by which aPL
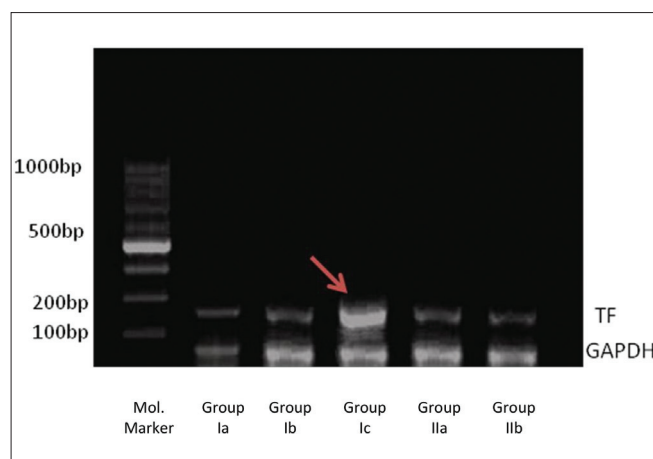
antibodies induce increased expression of TF on monocyte leads to oxidative burst, thrombosis, and fetal death.[8] In holding up the evidence, several autoantibodies, acting as functional inhibitors of anti-TF pathways, are detected in patients with APS that inhibits the function of TFPI.[15] An *in-vivo* experimental model of APS revealed that blockade of TF using anti-TF monoclonal antibody reduces inflammation, respiratory burst activity on neutrophils and fetal injury.[16] In addition, the over expression of TF is mainly due to cholesterol loading where oxidatively-modified low-density lipoprotein (ox-LDL) and cholesterol induces atherogenesis and TF expression.[6] Several novel statins were tested and found to play an inhibitory role on aPL-induced TF expression;[17] later, simvastatin[8] and fluvastatin[18] were demonstrated as reducing inflammation and fetal loss in APS-mediated mice.

We have already demonstrated that 6,7-dimethoxycoumarin, a hypolipidemic Chinese herb *Artemisia scoparia*, significantly reduces the risk of aCL-mediated fetal loss by

Table 2: Plasma enzyme levels in all the experimental and control groups

Plasma Enzymes	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)
Group Ia	153 ± 3.6	36.4 ± 8.3	47 ± 11.4	87.6 ± 2.6
Group Ib	131.6 ± 12.9	27.9 ± 3.9	53.1 ± 12.2	85.7 ± 3.2
Group Ic	171.4 ± 22.9*	45.9 ± 16.7	77.9 ± 21.1*	102.3 ± 4.8*
Group IIa	128.7 ± 14.7	31.1 ± 5.5	50.4 ± 17.1	85.6 ± 3.4
Group IIb	146.1 ± 18	35.3 ± 3.5	46.9 ± 6.9	84.7 ± 2.7

*$P < 0.05$ AST, ALP, and LDH levels were high in positive control mice population. Treatment with 6,7-Dimethoxycoumarin, after induction of experimental APS, significantly reduced AST, ALP and LDH levels in group Ia mice

Table 3: Cell count from all the experimental and control groups

Cells	WBC (cells/ µl)	RBC (million/µl)	Platelets (cells/ mm3x10-3)	Lymphocytes (%)
Group Ia	3500.1 ± 156.6	3.5 ± 0.0	1033 ± 60.9	44.6 ± 1.3
Group Ib	3549.9 ± 88.1	3.5 ± 0.1	1034.1 ± 41.6	45.9 ± 0.6
Group Ic	3594.6 ± 126.4	3.6 ± 0.1	846 ± 62.2*	46.3 ± 0.3
Group IIa	3609.6 ± 55.7	3.5 ± 0.1	1069.4 ± 79.5	46 ± 0.8
Group IIb	3573.9 ± 58.2	3.5 ± 0.0	1033.6 ± 57.5	45.9 ± 0.6

*$P < 0.05$, Reduction of platelet level was observed in group Ic i.e. positive control mice for APS when compared to all other groups. Treatment with 6,7-Dimethoxycoumarin after induction of APS significantly increased the platelet level in group Ia mice population
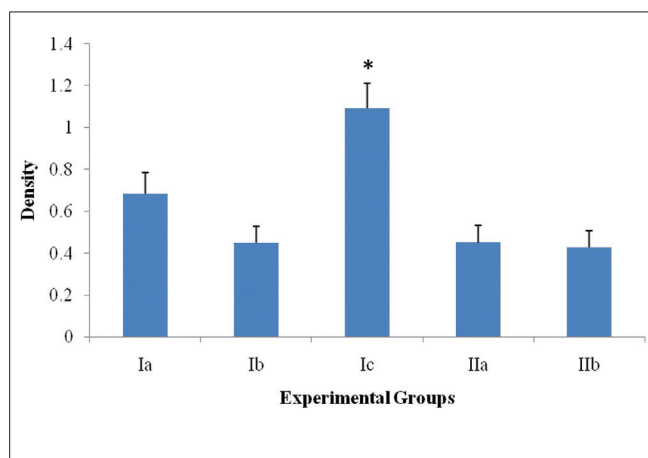

Figure 3: TF mRNA expression was high in group Ic mice i.e. positive control mice for APS. Following treatment with 6,7-dimethoxycoumarin after the induction of experimental APS, the expression of TF mRNA reduced in group Ia mice

Figure 4: Normalized ratios of TF mRNA/GAPDH mRNA in experimental and control groups. The ratio of TF mRNA/GAPDH mRNA expression was high in positive control mice for APS (group Ic). Treatment with 6,7-dimethoxycoumarin, after induction of experimental APS, significantly reduced the TF mRNA expression (group Ia)

ceasing TF expression on monocyte and placental cells to an extended level (unpublished data). The hypolipidemic and vaso-relaxant activity of 6,7-dimethoxycoumarin have already been extensively reported, and free radical scavenging activity is the backbone for the consequence of aforementioned activities.[12] Moreover, 6,7-dimethoxycoumarin is reported to have the property of reducing lipopolysaccharide-mediated TF expression on umbilical vein endothelial cells.[19] Based on the previous and present findings, we hypothesize that the hypolipidemic activity of the 6,7-dimethoxycoumarin may influence the over-expression of tissue factor in placenta at mRNA level in APS-mediated pregnancy loss. The results from the measurement of biochemical parameters of the present study reveal that 6,7-dimethoxycoumarin diminished LDL, cholesterol, and TF levels after the induction of experimental APS. Reduction of TF mRNA level in mice treated with 6,7-dimethoxycoumarin after the induction of APS is consistent with the earlier reports.

We noticed that significant increase in plasma enzymes, such as LDH, AST, ALP, and ALT, in aCL-induced positive control mice, suggest that destruction of RBC cells that might have caused by one of any predisposing factors such as the occurrence of autoimmune hemolytic anemia and elevated reactive oxygen species. In support of this, there are considerable evidences that prevalence of hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome in patients with APS and the incidence has been estimated 8/15 cases.[20] Our results also show a moderate increase in RBC levels in all the experimental and control groups after treatment with 6, 7-dimethoxycoumarin. We hypothesize that the reduction in RBC levels after treatment with 6,7-dimethoxycoumarin is due to its free radical scavenging and hepatoprotecive activity.[21] It has been observed that enhanced TF expression on the monocytes in response to activated platelets during oxidative stress condition, followed by supplementation of antioxidants significantly decreased monocytes TF antigen and its activity.[22] 6,7-dimethoxycoumarin has already been proven that it has the capability of reducing superoxide anion-mediated TF expression and avert disseminated intravascular coagulation.[19] It is clear from our study that 6,7-dimethoxycoumarin significantly reduced aCL antibody-mediated TF overexpression in placenta, and increased the platelet count to be comparable with negative control mice (normal level).

CONCLUSION

The present study concludes that the 6,7-dimethoxycoumarin has the capability of reducing TF expression on placenta at the mRNA level and thrombus generation indirectly by its potent anti-TF and anti-oxidant activities. Moreover, the hypolipidemic property of the 6,7-dimethoxycoumarin can potentially reduce cholesterol and ox-LDL levels in APS.

ACKNOWLEDGEMENT

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