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Dietary gamma oryzanol plays a significant role in the anti-inflammatory activity of rice bran oil by decreasing pro-inflammatory mediators secreted by peritoneal macrophages of rats

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ABSTRACT

Ricebran oil (RBO) is promoted as heart friendly oil because of its ability to maintain serum lipids at desirable levels. Inflammation also plays an important role on cardiovascular health. The role of minor constituents present in unsaponifiable fraction (UF) of RBO on inflammatory markers is not well understood. To evaluate this, we have taken RBO with UF (RBO-N), RBO stripped of UF (RBO-MCR) and RBO-MCR supplemented with UF from RBO (UFRBO) or Gamma-Oryzanol (γ-ORY) were added in AIN-93 diets which was then fed to Wistar rats for a period of 60 days. Groundnut oil with UF (GNO-N), UF removed GNO (GNO-MCR) and GNO-MCR supplemented with UF from RBO or γ-ORY was also used for comparison. The peritoneal macrophages from the rats were activated and pro-inflammatory mediators such as Reactive Oxygen Species (ROS), eicosanoids, cytokines, hydrolytic enzymes of lysosomal origin were monitored. The results indicated that UF of RBO and γ-ORY supplemented in the dietary oils play a significant role in reducing the secretion of pro-inflammatory mediators by macrophages. Hence γ-ORY in RBO significantly contributed to the anti-inflammatory properties of RBO.

1. Introduction

Dietary oils are mixtures of Triacylglecerol (TAG) which forms the bulk of the oil. In addition, minor constituents like phospholipids, free fatty acids, phytosterols, glycolipids, sphingolipids and many other nutraceutical molecules are present in the unsaponifiable fraction (UF) of the oil. These minor constituents are removed when the UF was stripped from the oil during refining process. Dietary oils have significant impact in controlling the serum lipid levels and pro-inflammatory mediators which are risk factor makers for cardiovascular disease [1]. Very often the effects of dietary oils on serum lipid levels are attributed to the overall fatty acid composition as well as to the positional distribution of these fatty acids in the TAG molecules [2]. The contributions of minor constituents towards health benefits of oils and fats are seldom recognized [3,4]. Rice bran oil (RBO) contains a unique minor constituent, Gamma-Oryzanol (γ-ORY) in the UF which provides health benefits. RBO is widely used for cooking purposes and is promoted as health oil in Japan and India [4,5]. It exhibits antioxidant, anti-inflammatory, anti-thrombotic, and hypocholesterolemic effects [4–8]. We have recently demonstrated that antioxidant activity of RBO is greatly reduced when UF of RBO was stripped from the oil [9]. However, the ability of minor constituent removed RBO (RBO-MCR) to impact fatty acid composition of serum and tissue lipids was not altered. The impact of feeding RBO-MCR on the levels of pro-inflammatory mediators like Reactive Oxygen Species (ROS), eicosanoids, cytokines and hydrolytic enzyme was addressed in this study. These parameters have an impact on the cardiovascular health.

Activated macrophages generate number of pro-inflammatory mediators. The generation of these pro-inflammatory mediators in an uncontrolled manner can lead to chronic diseases. The present investigation is focused on evaluating the effect of dietary RBO, RBO-MCR and RBO-MCR supplemented with γ-ORY on the secretion of pro-inflammatory mediators by peritoneal macrophages in rats.

Abbreviations: GNO, Groundnut oil; RBO, Rice bran oil; MCR, Minor constituents removed oil (Unsaponifiable fraction removed oil); N, Native oil (with unsaponifiable fraction); ROS, Reactive oxygen species; UFRBO, Unsaponifiable fraction from RBO; γ-ORY, Gamma-Oryzanol; UF, Unsaponifiable fraction.

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2. Materials and methods

2.1. Materials

RPMI (Roswell Park Memorial Institute)-1640, 12- well coastar plates, antibiotic solutions (penicillin & streptomycin) and supplements used in the diet preparation were purchased from Himedia, Mumbai, India. Foetal calf serum, heparin, superoxide dismutase, phorbol myristate acetate (PMA), ferricytochrome C, zymosan, hyaluronic acid, calciumionophore, lipopolysaccharide, sulphanailamide, sodium nitrite and DNA were purchased from Sigma Chemical Company, St. Louis, Mo. USA. Standards for eicosanoids were obtained from Cayman chemicals, USA. Groundnut oil (GNO) and Ricebran Oil (RBO) of reputed brands were purchased from a local super market, Mysuru, India. γ-Oryzanol (>99% purity) was a generous gift from Amohusu Chemical Industries Inc, Tokyo, Japan.

2.2. Experimental animals

Male Wistar rats (Out breed- Wistar, IND- cft (2c)) (Rattus norvegicus) were used for all the experiments. The experimental protocol was approved by Institute Animal Ethical Committee (Reg. No: 49/1999/CPCSEA) of CSIR- CFTRI, Mysuru, India. The rats were housed in individual cages and temperature of the room was maintained at 25 °C with a 12 h light/dark cycle in an approved animal house facility at Central Food Technological Research Institute, Mysuru, India. Weaning rats (50–60 g) were fed AIN-93 purified diet [10] supplemented with one of the following lipids: RBO with UF (RBO-N), RBO in which UF was removed (RBO-MCR), RBO-MCR to which UF from RBO was added back (RBO-MCR + UFRBO) and RBO-MCR to which γ-ORY was added at 700 mg/kg diet (RBO-MCR+γ-ORY). In addition, diet containing Groundnut oil with UF (GNO-N), GNO in which UF was removed (GNO-MCR), GNO-MCR to which UF from RBO was added (GNO-MCR + UFRBO) and GNO-MCR to which γ-ORY added at 700 mg/kg diet (GNO-MCR+γ-ORY) was also used. The final concentration of the fat in the diet was maintained at 10 wt%. The basal composition of the diet (g/kg diet) was corn starch; 500, casein; 200, sucrose; 100, fat; 100, cellulose; 50, mineral mix; 35, vitamin mix; 10, L-cysteine; 3, choline bitartarate; 2. The diets were fed adlibitum to rats for a period of 60 days. The animals had free access to drinking water.

2.3. Analytical methods

2.3.1. Cell culture

Peritoneal macrophages from rats were aspirated in 30 mL of Hank's balanced salt solution (HBSS) containing 10 IU heparin per mL according to the method of Rao & Lokesh [11]. Peritoneal cells were centrifuged at 600 × g at 4 °C for 8min and suspended in RPMI- 1640 containing 2% foetal calf serum. The cells (2.5×10^6 cells/mL) were plated on 12 well coastar plates and incubated for 3 h at 37 °C in a humidified incubator with 5% CO_2. The adhering macrophages were washed thrice with HBSS. Viability of macrophages was greater than 95% as judged by the exclusion of trypan blue.

2.3.2. Reactive oxygen species

The adhering macrophages were incubated with 80 μM ferricytochrome C, 10 μM PMA in the presence and absence of SOD (10 μM). Final volume was made up to 1 mL in HBSS. Culture plates were kept in an incubator at 37 °C with 5% CO_2 for 60 min. The supernatant was aspirated and the absorbance was measured at 550 nm [12]. The superoxide anion ($O_2^{\cdot-}$) generation was measured as the superoxide dismutase (SOD) inhibited reduction of ferricytochrome C. Nitric oxide ($NO^{\cdot}$) was quantitated in terms of nitrite formed [13] after activating macrophages with LPS (20 μg/mL) for 16 h. Sodium nitrite was used as reference standard (1–100 μM). The results were expressed as nmoles of nitrite formed per μg of macrophage DNA.

2.3.3. Eicosanoids

The macrophages were incubated with 2 mmol/L of $CaCl_2$ and 0.5 mmol/L of $MgCl_2$ in HBSS at 37 °C and 5% CO_2 [11]. The cells were stimulated with calcium ionophore (0.5 μM) for 15 min. The final volume was maintained at 2 mL with HBSS. The reaction was stopped by adding 1 mL of ice cold methanol and the solution was adjusted to pH 3.0 with 3% formic acid. The cells were sonicated and centrifuged. The cell supernatant was passed through Sep Pak C-18 column (Waters, Millipore Corp., Milford, MA, USA) and eluted using ethyl acetate. The ethyl acetate extracts were loaded on RP-C-18 column (Supelco, Discovery, 5 μM, 25 cm × 4.6 mm, Bellefonte, USA) fitted to a SPD- 20A HPLC (Shimadzu Corp., Tokyo, Japan). The prostaglandins and thromboxanes were eluted with methanol: water: trifluoroacetic acid: triethylamine (80:20:0.1:0.05 v/v) at a flow rate of 0.7 mL/min and monitored at 200 nm [14]. The leukotrienes were eluted with acetonitrile: methanol: acetic acid: water (65:10:1:24 v/v) adjusted to pH 5.6 with ammonia and monitored at 280 nm [15]. The eicosanoids were quantified by using reference standards (Cayman chemicals, USA).

2.3.4. Cytokines

Two mL of fresh medium (RPMI-1640) was added to the adhered macrophages and incubated with LPS (5 μg/mL) for 24 h at 37 °C. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL- 6), interleukin-4 (IL-4) and interleukin-10 (IL-10) from culture supernatant was estimated using solid phase sandwich enzyme linked immunosorbant assay (ELISA) kits (Invitrogen Corporation, Camarillo, CA). The cytokines were analyzed by ELISA following the manufacturer's instructions for using the kits. 100 μL of culture supernatants were centrifuged at 1000 × g for 3min and applied to a 96 well micro titer plate pre-coated with specific monoclonal antibody. The ELISA plates were read at 450 nm in ELISA reader Infinite M200 PRO, Tecan Austria GmbH, Austria.

2.3.5. Lysosomal enzymes

The adhered macrophages were activated with zymosan A (1 mL HBSS containing 500 μg zymosan A) for 24 h. The collagenase and elastase activities were determined from culture supernatants using EnzChek®Collagenase Assay kit and EnzChek®Elastase Assay kit, respectively (Invitrogen GmbH, Darmstadt, Germany) in a micro plate reader (Infinite M200 PRO, Tecan Austria GmbH, Austria) set for excitation at 485 ± 10 nm and emission detection at 530 ± 15 nm. Hyaluronidase activity was determined by the amount of N-acetyl glucosamine released from hyaluronic acid [16,17].

2.3.6. Quantification of macrophage DNA

Macrophage DNA was quantified using calf thymus, Type-1 DNA as reference standard [18].

2.3.7. Estimation of protein

Macrophage protein was quantified by the method of Lowry et al. [19] after digesting the cells in 1 mL of 1 N NaOH overnight. The spectrophotometric measurements were carried out in a Shimadzu ultraviolet 1601A spectrophotometer (Shimadzu, Kyoto).

2.4. Statistical analysis

Results are presented as mean ± standard deviation for each group. The data was analyzed by one-way ANOVA followed by a post hoc Tukey test to compare the control and treatment groups; *P*-values of less than 0.05 were considered as statistically significant. All statistical analysis was performed using SPSS statistical software package version 17.0.

3. Results

3.1. Effect of dietary lipids on generation of ROS by macrophages

The dietary lipids significantly influenced the levels of ROS secreted by macrophages. The macrophages from rats fed a diet containing RBO-N generated lower levels of $O_2^{\cdot-}$ and $NO^{\cdot}$ by 51 and 45%, respectively as compared to those from rats fed GNO-N (Table 1). But the macrophages from rats fed RBO-MCR generated lower levels of $O_2^{\cdot-}$ and $NO^{\cdot}$ by 16 and 8%, respectively as compared to those from rats fed GNO-N. This indicated that the removal of UF from RBO significantly compromised their ability to reduce the generation of ROS by macrophages. The UF which was removed from RBO, when added back restored the ability of the dietary RBO to reduce the generation of ROS by macrophages. The macrophages form rats fed diet containing RBO-MCR supplemented with UF from RBO showed reduced generation of $O_2^{\cdot-}$ by 37 and $NO^{\cdot}$ by 35% respectively as compared to macrophages from rats which were given diet containing RBO-MCR. Therefore, the UF of RBO significantly contributed to the suppression of the ROS generation by macrophages. γ-ORY is a major component of UF from RBO. The addition of γ-ORY to diet containing RBO-MCR also showed reduced ROS generation by macrophages to an extent of 28 and 27% respectively for $O_2^{\cdot-}$ and $NO^{\cdot}$. This indicated that nearly 73–77% of the effect of RBO in the suppression of ROS generation in macrophages could be attributed to γ-ORY present in the UF of the oil. The macrophages from rats fed GNO-N or GNO-MCR showed no significant differences in the generation of ROS indicating that the components present in UF of GNO has no impact on ROS generation. However, rats fed a diet containing GNO-MCR to which UF from RBO was added showed a significant reduction in the generation of $O_2^{\cdot-}$ and $NO^{\cdot}$ to an extent of 33 and 34% respectively indicating that UF from RBO suppress ROS generation irrespective of the oil (GNO-MCR or RBO-MCR) to which it was supplemented. Here again rats fed a diet containing γ-ORY to GNO-MCR showed reduced generation of ROS by macrophages to an extent of 25 and 27% respectively as compared to that observed in rats fed diet containing GNO-MCR (Table 1).

Table 1
Effect of dietary lipids on secretion of ROS by peritoneal macrophages in rats.

Dietary lipids	$O_2^{\cdot-}$	$NO^{\cdot}$
	(n mol/μg DNA)	
RBO-N	22.7 ± 2.1^{a}	15.7 ± 1.2^{a}
RBO-MCR	39.2 ± 2.8^{c}	26.5 ± 2.2^{d}
RBO-MCR + UFRBO	24.7 ± 2.1^{a}	17.2 ± 1.6^{ab}
RBO-MCR+ γ-ORY	28.2 ± 2.4^{ab}	19.3 ± 1.7^{b}
GNO-N	46.8 ± 3.0^{d}	28.7 ± 2.2^{de}
GNO-MCR	49.7 ± 3.1^{d}	30.8 ± 2.4^{e}
GNO-MCR + UFRBO	33.5 ± 2.7^{b}	20.2 ± 1.6^{bc}
GNO-MCR+γ-ORY	37.0 ± 2.6^{bc}	22.4 ± 2.0^{c}

RBO-N: Rice bran Oil with UF, GNO: Groundnut Oil, N: Oil containing UF, MCR: Minor Constituents Removed (UF removed oil), UFRBO: UF from RBO added, γ-ORY: γ-Oryzanol. $O_2^{\cdot-}$: Superoxide anions, NO: Nitric oxide. Each value represents the mean ± SD, n = 6 rats per group. Values in a column with same superscript are not statistically significant at $P < 0.05$.

3.2. Effect of dietary lipids on secretion of eicosanoids by macrophages

The macrophages from rats fed diet containing RBO-N secreted lower levels of PGE_2, TXB_2, LTB_4 and LTC_4 by 49, 34, 38 and 47%, respectively as compared to those from rats fed GNO-N (Table 2). The macrophages from rats fed diet containing RBO-MCR secreted lower levels of PGE_2 by 17% as compared to macrophages from rats fed GNO-N. However, no significant differences were observed in the secretion of TXB_2, LTB_4 and LTC_4 in rats fed diet containing RBO-MCR and GNO-N or GNO-MCR. This indicated that the ability of RBO-N to influence eicosanoid secretion by macrophages is compromised when UF is removed from the RBO-N. 6-keto $PGF_{1\alpha}$ secretion by macrophages was however enhanced by 36% in rats fed diet containing RBO-N as compared to those given GNO-N. The macrophages from rats fed RBO-MCR to which UF from RBO was added back secreted lower levels of PGE_2, TXB_2, LTB_4 and LTC_4 by 32, 31, 33 and 31%, respectively as compared to macrophages from rats fed RBO-MCR. This indicated that the ability of dietary RBO to lower eicosanoid secretion by macrophages is significantly influenced by the components present in UF. This is substantiated by the finding that the macrophages from rats fed RBO-MCR+γ-ORY secreted lower levels of PGE_2, TXB_2, LTB_4 and LTC_4 by 28, 27, 26 and 27%, respectively as compared to those from rats fed RBO-MCR. This indicated that γ-ORY per se contributed to the ability of dietary RBO to suppress eicosanoid formation to an extent of 86%. Here again the UF from GNO did not show any impact on eicosanoid formation by macrophages. However, rats fed diet containing GNO with added UF from RBO secreted significantly lower amounts of PGE_2, TXB_2, LTB_4 and LTC_4 by 35, 35, 36 and 32%, respectively as compared to that secreted by macrophages from rats fed GNO. The macrophages from rats fed diet containing GNO-MCR+ γ-ORY secreted lower levels of PGE_2, TXB_2, LTB_4 and LTC_4 by 28, 27, 28 and 25%, respectively as compared to that observed in rats fed GNO. Therefore, the components from UF of RBO, particularly γ-ORY contributed significantly to lower the secretion of pro-inflammatory eicosanoids by macrophages (Table 2).

3.3. Effect of dietary lipids on secretion of cytokines by macrophages

The macrophages from rats fed a diet containing RBO secreted decreased levels of pro-inflammatory cytokines like TNF-α and IL-6 by 65 and 40%, respectively as compared to those from rats fed GNO (Table 3). The macrophages from rats fed diet containing UF removed RBO secreted lower levels of TNF-α and IL-6 by 41 and 3%, respectively as compared to those from rats fed GNO. The macrophages from rats fed RBO-MCR + UFRBO secreted lower levels of TNF-α and IL-6 by 33 and 32%, respectively as compared to those from rats fed RBO-MCR. The macrophages from rats fed RBO-MCR+γ-ORY secreted lower levels of TNF-α and IL-6 by 26 and 26%, respectively as compared to those from rats fed RBO-MCR. These studies indicated that removal of UF from RBO (RBO-MCR) significantly reduces its ability to lower the secretion of pro-inflammatory cytokines by macrophages. This however could be partly reversed by adding the UF back to RBO-MCR. Addition of γ-ORY to RBO-MCR restored its ability to lower pro-inflammatory cytokines to an extent of 80%.

The macrophages from rats fed a diet containing RBO secreted higher levels of anti-inflammatory cytokines like IL-4 and IL-10 by 134 and 143%, respectively as compared to those from rats fed GNO (Table 3). The macrophages form rats fed RBO-MCR showed enhanced levels of IL-4 and IL-10 by only 45 and 43%, respectively,

Table 2
Effect of dietary lipids on secretion of eicosanoids by peritoneal macrophages in rats.

Dietary lipids	Eicosanoids (ng/mg protein)				
	PGE_2	6- keto $PGF_{1\alpha}$	TXB_2	LTB_4	LTC_4
RBO-N	18.2 ± 1.6^{a}	24.9 ± 1.7^{d}	30.6 ± 2.4^{a}	35.6 ± 2.7^{a}	26.9 ± 2.3^{a}
RBO-MCR	29.4 ± 1.9^{d}	14.8 ± 1.2^{a}	49.3 ± 2.8^{c}	58.9 ± 3.1^{bc}	44.3 ± 2.6^{cd}
RBO-MCR + UFRBO	20.0 ± 1.7^{ab}	22.1 ± 1.9^{cd}	33.8 ± 2.5^{ab}	39.7 ± 2.9^{ab}	30.4 ± 2.3^{ab}
RBO-MCR+γ-ORY	21.3 ± 1.8^{b}	20.2 ± 1.6^{c}	36.1 ± 2.6^{b}	43.8 ± 3.0^{b}	32.2 ± 2.2^{b}
GNO-N	35.5 ± 2.3^{e}	15.8 ± 1.3^{a}	46.4 ± 2.9^{c}	57.4 ± 3.4^{bc}	50.4 ± 2.6^{d}
GNO-MCR	37.4 ± 2.4^{e}	14.3 ± 1.1^{a}	49.1 ± 3.0^{c}	60.8 ± 3.7^{c}	53.9 ± 3.0^{d}
GNO-MCR + UFRBO	24.2 ± 2.1^{bc}	19.4 ± 1.4^{b}	32.0 ± 2.7^{a}	39.2 ± 3.0^{ab}	36.8 ± 2.7^{bc}
GNO-MCR+γ-ORY	26.9 ± 2.2^{c}	17.4 ± 1.3^{ab}	35.6 ± 2.8^{b}	43.5 ± 3.2^{b}	40.3 ± 2.8^{c}

RBO-N: Rice bran Oil with UF, GNO: Groundnut Oil, N: Native, MCR: Minor Constituents Removed (UF removed oil), UFRBO: UF from RBO added, γ-ORY: γ-Oryzanol. PGE_2: Prostaglandin E_2, TXB_2: Thromboxane B_2, LTB_4: Leukotriene B_4. Each value represents the mean ± SD, n = 6 rats per group. Values in a column with same superscript are not statistically significant at $P < 0.05$.

when compared to those from rats fed GNO. The macrophages from rats fed RBO-MCR + UFRBO secreted higher levels of IL-4 and IL-10 by 109 and 114%, respectively as compared to those from rats fed GNO. The macrophages from rats fed RBO-MCR+γ-ORY secreted higher levels of IL-4 and IL-10 by 98 and 100%, respectively as compared to those from rats fed GNO-N. This again indicated that the components present in UF of RBO of which γ-ORY significantly contributed to the anti-inflammatory effects of RBO.

The macrophages from rats fed a diet containing GNO-MCR secreted similar levels of TNF-α and IL-6 as compared to those from rats fed GNO-N (Table 3). The macrophages from rats fed GNO-MCR + UFRBO however secreted lower levels of TNF-α and IL-6 by 27 and 31%, respectively as compared to those from rats fed GNO. The macrophages from rats fed GNO-MCR+γ-ORY secreted lower levels of TNF-α and IL-6 by 19 and 31%, respectively as compared to those from rats fed GNO.

The macrophages from rats fed a diet containing GNO-MCR secreted similar levels of IL-4 and IL-10 as compared to those from rats fed GNO (Table 3). There was a marked increase in the secretion of IL-4 and IL-10 by 25 and 24% respectively by macrophages from rats fed GNO-MCR + UFRBO as compared to those from rats fed GNO. The macrophages from rats fed GNO-MCR+γ-ORY secreted higher levels of IL-4 and IL-10 by 20 and 14%, respectively as compared to those from rats fed GNO. Thus the UF of RBO or γ-ORY which is the major UF component of RBO lowered the secretion of pro-inflammatory cytokines and also enhanced the secretion of anti-inflammatory cytokines irrespective of the oils (GNO or RBO) they were introduced for feeding rats.

Table 3
Effect of dietary lipids on secretion of cytokines by peritoneal macrophages in rats.

Dietary lipids	Pro-inflammatory cytokines		Anti-inflammatory cytokines	
	(ng/mg protein)			
	TNF-α	IL-6	IL-4	IL-10
RBO-N	5.8 ± 0.4^{a}	6.2 ± 0.5^{a}	12.9 ± 1.0^{d}	5.1 ± 0.4^{e}
RBO-MCR	9.7 ± 0.7^{b}	10.1 ± 0.8^{c}	8.0 ± 0.5^{c}	3.0 ± 0.2^{c}
RBO-MCR + UFRBO	6.5 ± 0.6^{a}	6.9 ± 0.3^{a}	11.5 ± 0.9^{d}	4.5 ± 0.3^{de}
RBO-MCR+γ-ORY	7.2 ± 0.5^{ab}	7.5 ± 0.6^{ab}	10.9 ± 0.8^{cd}	4.2 ± 0.2^{d}
GNO-N	16.5 ± 1.2^{d}	10.4 ± 0.8^{c}	5.5 ± 0.3^{a}	2.1 ± 0.1^{a}
GNO-MCR	18.2 ± 1.4^{d}	11.2 ± 0.9^{c}	5.0 ± 0.3^{a}	1.9 ± 0.1^{ab}
GNO-MCR + UFRBO	12.0 ± 1.1^{c}	7.2 ± 1.5^{a}	6.9 ± 0.5b	2.6 ± 0.1^{b}
GNO-MCR+γ-ORY	13.4 ± 1.2^{c}	8.1 ± 1.6^{b}	6.6 ± 0.4^{bc}	2.4 ± 0.1^{ab}

RBO-N: Rice bran Oil with UF, GNO: Groundnut Oil, N: Native, MCR: Minor Constituents Removed (UF removed oil), UFRBO: UF from RBO added, γ-ORY: γ-Oryzanol. IL: Interleukin, TNF-α: Tumor Necrosis Factor-α. Each value represents the mean ± SD, n = 6 rats per group. Values in a column with same superscript are not statistically significant at $P < 0.05$.

Table 4
Effect of dietary lipids on secretion of lysosomal enzymes by peritoneal macrophages inn rats.

Dietary lipids	Lysosomal enzymes (U/mg protein)		
	Collagenase	Elastase	Hyaluronidase
RBO-N	0.22 ± 0.01^{a}	0.32 ± 0.03^{a}	1.3 ± 0.1^{a}
RBO-MCR	0.37 ± 0.03^{c}	0.53 ± 0.04^{d}	2.2 ± 0.2^{c}
RBO-MCR + UFRBO	0.24 ± 0.01^{ab}	0.35 ± 0.04^{ab}	1.5 ± 0.2^{ab}
RBO-MCR+γ-ORY	0.27 ± 0.02^{b}	0.38 ± 0.05^{b}	1.6 ± 0.3^{b}
GNO-N	0.38 ± 0.03^{c}	0.56 ± 0.06^{de}	2.9 ± 0.2^{d}
GNO-MCR	0.41 ± 0.05^{d}	0.59 ± 0.07^{e}	3.2 ± 0.3^{d}
GNO-MCR + UFRBO	0.28 ± 0.04^{b}	0.38 ± 0.04^{b}	2.1 ± 0.1^{c}
GNO-MCR+γ-ORY	0.30 ± 0.02^{b}	0.43 ± 0.05^{c}	2.3 ± 0.2^{c}

RBO-N: Rice bran Oil with UF, GNO: Groundnut Oil, N: Native, MCR: Minor Constituents Removed (UF removed oil), UFRBO: UF from RBO added, γ-ORY: γ-Oryzanol. Each value represents the mean ± SD, n = 6 rats per group. Values in a column with same superscript are not statistically significant at $P < 0.05$.

3.4. Effect of dietary lipids on secretion of lysosomal enzymes by macrophages

Lysosomal enzymes secreted by macrophages also contribute to pro-inflammatory responses in host. The macrophages from rats fed diet containing RBO secreted lower levels of collagenase, elastase and hyaluronidase by 42, 43 and 55%, respectively as compared to those from rats fed diet containing GNO (Table 4). The macrophages from rats fed RBO-MCR secreted lower levels of collagenase, elastase and hyaluronidase by 0, 5 and 24%, respectively as compared to those from rats fed GNO. The macrophages from rats fed RBO-MCR + UFRBO secreted lower levels of collagenase, elastase and hyaluronidase by 37, 37 and 48%, respectively as compared to those from rats fed GNO. The macrophages from rats fed RBO-MCR+γ-ORY also secreted lower levels of collagenase, elastase and hyaluronidase by 29, 32 and 45%, respectively as compared to those from rats fed GNO.

The macrophages from rats fed GNO-MCR secreted similar levels of collagenase, elastase and hyaluronidase as compared to those from rats fed GNO (Table 4). The macrophages from rats fed GNO-MCR + UFRBO secreted lower levels of collagenase, elastase and hyaluronidase by 26, 32 and 28%, respectively as compared to those from rats fed GNO. The macrophages from rats fed GNO-MCR+γ-ORY secreted lower levels of collagenase, elastase and hyaluronidase by 21, 23 and 21%, respectively as compared to those from rats fed GNO.

4. Discussion

The primary objective of this investigation was to assess the role

of bioactive components of RBO with particular reference to γ-ORY in regulating the levels of pro-inflammatory mediators like ROS, eicosanoiods, cytokines and hydrolytic enzymes of lysosomal origin secreted by rat peritoneal macrophages. This was assessed by feeding rats with RBO, RBO in which minor constituents were removed (RBO-MCR, by stripping unsaponifiable fraction), RBO-MCR to which UF or γ-ORY was added back and monitoring the secretion of pro-inflammatory mediators having a bearing on cardiovascular health. A similar study was also made by feeding rats with GNO, GNO-MCR, GNO-MCR + UFRBO and GNO-MCR+γ-ORY. It should be mentioned here that both RBO and GNO has similar fatty acid composition, but differ in the composition of minor constituents present in the UF of the oil. RBO contains 1.2–1.4 g of γ-ORY per 100 g oil. GNO did not contain γ-ORY or any other identifiable specific minor constituents.

The major findings of the study are as follows. The macrophages isolated form rats fed RBO secreted decreased levels of pro-inflammatory compounds as compared to that observed from rats fed GNO. Removal of UF from RBO before it was fed to rats significantly decreased the ability of RBO to lower the secretion of inflammatory compounds. However, removal of UF from GNO before it was fed to rats has no impact on the secretion of inflammatory mediators by macrophages. This indicated that UF of RBO contributed significantly to lowering the levels of pro-inflammatory mediators in rats. However, the UF from GNO had no impact on the secretion of inflammatory mediators in rats. Further, the UF from RBO added back to RBO-MCR or GNO-MCR significantly decreased the secretion of inflammatory markers by macrophages. This indicated that minor constituents present in UF of RBO per se have anti-inflammatory properties. This is observed irrespective of whether it was added to GNO or RBO. The addition of equivalent amounts of γ-ORY that was present in the UF of RBO to RBO-MCR or GNO-MCR indicated that nearly 70–85% of the anti-inflammatory nature of dietary RBO could be accounted by γ-ORY. Therefore, γ-ORY present in the UF could be considered as the nutraceutical molecule which is contributing to anti-inflammatory activity of RBO. It is also interesting to note that the effect of RBO-MCR on the secretion of $O_2^{\cdot-}$, $NO^{\cdot}$, TXB_2, LTB_4, LTC_4, IL-6 and lysosomal enzyme secretion by macrophages are comparable to that observed in macrophages from rats fed GNO. This indicated a significant role for γ-ORY in RBO towards the anti-inflammatory properties of dietary RBO. Earlier we had demonstrated that the components of UF in RBO significantly influenced the total antioxidant activity in rats as well as in lowering Cu^{2+} induced LDL oxidation [9]. However, the UF of RBO had no influence on fatty acid composition of serum and tissue lipids compared to that observed with dietary RBO.

The mechanism by which γ-ORY per se exhibits its anti-inflammatory activities has received less attention. It is known that the activation of Nuclear Factor- kappa B (NF-κB) triggers the pro-inflammatory signaling pathway leading to over production of ROS, lysosomal enzymes, eicosanoids, cytokines and matrix metallo proteases by macrophages [20]. Recently, we have demonstrated that the secretion of IL-1β by peritoneal macrophages in rats fed RBO containing γ-ORY is significantly reduced as compared to those fed hydrogenated fat [21]. Further, dietary RBO also up-regulated the expression of Adiponectins and down-regulated the expression of Toll Like Receptors (TLR-2 and TLR-4) and NF-κBp65 in macrophages [21]. Our study indicated that the anti-inflammatory properties of RBO may be mediated by lowering the levels of NF-κBp65 which decreases the levels of pro-inflammatory mediators. Studies have shown the hepato protective effect of ferulic acid in carbon tetrachloride induced acute liver injury [22]. carbontetrachloride treatment increased the expressions of TNF-α, inducible nitric oxide synthase, Cyclooxygenase-2, NF-κB, TLR2 and TLR4. Ferulic acid significantly attenuated the levels of these pro-inflammatory mediators in the mice [22]. Rondini et al. [23] observed a significant elevation in antioxidant activity in rats fed diet containing ferulic acid. γ-ORY is a mixture of ferulic acid esters and our results on the anti-inflammatory effects of ferulic acid derivatives are in agreement with findings of Islam et al. [22] and Rondini et al. [23].

Several studies have shown that the unique minor constituents in selected oils provide stability and health benefits [24]. It is advisable to optimize the refining methods for these oils to retain minor constituents in sufficient amounts which can be utilized for preparing functional foods. The commercially available vegetable oils are subjected to regulatory guidelines under the Food Safety and Standards Act of India [25]. Accordingly, it is specified that the UF in GNO should not be more than 1% and that of RBO should not be more than 4.5%. RBO used in the present study contained UF to an extent of up to 4.5% in which γ-ORY was present to an extent of 1.4%. The amount of γ-ORY present in physically refined RBO was sufficient to exhibit the anti-inflammatory effects. This justifies promoting the physically refined RBO with γ-ORY for cardiovascular health as well as to curtail pro-inflammatory mediators.

Conflict of interest

There is no conflict of interest.

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