

Cocoa and Cocoa Fibre Intake Modulate Reactive Oxygen Species and Immunoglobulin Production in Rats Submitted to Acute Running Exercise [†]

Patricia Ruiz-Iglesias ^{1,2}, Raquel Gómez-Bris ¹, Malén Massot-Cladera ^{1,2},
María J. Rodríguez-Lagunas ^{1,2}, Francisco J. Pérez-Cano ^{1,2,*} and Margarida Castell ^{1,2,*}

¹ Section of Physiology, Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science, University of Barcelona (UB), 08028 Barcelona, Spain; patriciaruiz@ub.edu (P.R.-I.); raquel.gb96@gmail.com (R.G.-B.); malen.massot@ub.edu (M.M.-C.); mjrodriguez@ub.edu (M.J.R.-L.)

² Nutrition and Food Safety Research Institute (INSA-UB), Torribera Campus, Faculty of Pharmacy and Food Science, UB, 08921 Santa Coloma de Gramenet, Spain

* Correspondence: franciscoperez@ub.edu (F.J.P.-C.); margaridacastell@ub.edu (M.C.)

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Abstract: Acute high-intensity exercise can impair the immune system, and lead to oxidative stress. Cocoa intake might help in protecting against oxidative damage and impaired immune functioning. The aim of this study was to establish the effect of cocoa and cocoa fibre on the oxidative status and the immunoglobulin (Ig) production of rats following a bout of acute exercise on a treadmill. The production of reactive oxygen species (ROS) by macrophages and the concentration of serum and mucosal Ig was assessed 16 h after the running session. Exercise increased ROS production and decreased the serum IgG concentration and the salivary gland IgM content. A cocoa fibre-enriched diet prevented the increased ROS production and the reduction in salivary IgM induced by exercise, although it decreased the IgA content in serum and the salivary glands. Overall, cocoa, by means of its fibre content, can partially prevent the alterations in ROS and Ig production induced by a single session of intensive running exercise.

Keywords: exercise; antioxidant; ROS; oxidative stress; immunoglobulin; immune system; mucosal immunity; cocoa; cocoa fibre

1. Introduction

Although regular bouts of moderate-intensity exercise provide several health benefits, acute high-intensity exercise can impair the immune system, particularly the mucosal immune system [1], and lead to oxidative stress [2]. Due to its content of polyphenols and other bioactive compounds, cocoa intake might help protecting against the oxidative damage and the impaired immune function [3]. Moreover, cocoa fibre, by influencing the intestinal immune system [4], could also play a protective role.

2. Materials and Methods

2.1. Animals

Four-week-old female Wistar rats (N = 48) were purchased from Envigo (Huntingdon, United Kingdom) and maintained in the animal facilities of the Faculty of Pharmacy and Food Science at the University of Barcelona under controlled temperature and humidity conditions, in a 12/12h

light/dark cycle. Animals were housed in polycarbonate cages (4 rats per cage) and were given ad libitum access to food and water. All animal procedures were approved by the Ethical Committee for animal Experimentation of the University of Barcelona and the Catalonia Government (CEEA/UB ref. 517/18).

2.2. Acute Exercise Protocol and Diets

Exercise was performed on a treadmill for rodents with electric shock stimulation (Exer3/6, Columbus, OH, USA). At the beginning of the study, rats were adapted to the treadmill for 1 week by increasing time and speed. Then, all rats performed a pre-selection exhaustion test with an initial speed of 18 m/min with increasing increments of 3 m/min every 2 min until exhaustion, which was defined as the time that rats remained at the end of the treadmill despite the shock stimulation. From the physical performance in the pre-selection exhaustion, rats were randomly distributed into three dietary groups (n = 8/each): reference (REF), 10% cocoa (C10) and 5% cocoa fibre (CF). The reference group (REF) was fed with a standard AIN-93M diet (Envigo); the cocoa group (C10) received a special chow containing 10% cocoa (Idilia Foods S.L., Barcelona, Spain) and the 5% cocoa fibre group (CF) was fed with a diet containing the same fibre proportion as C10 diet but with a lower amount of polyphenols (Table 1), as in previous studies [4].

Table 1. Composition of the experimental diets.

Components	REF Diet (g/kg)	CF Diet (g/kg)	C10 Diet (g/kg)
Nutrients provided by the basal mix:			
Casein	140.0	113.3	123.6
L-cystein	1.8	1.7	1.96
Maize starch	465.7	402.5	484.0
Maltodextrin	155.0	142.6	168.6
Sucrose	100.0	96.8	109.1
Soyabean oil	40.0	36.3	31.4
Cellulose	50.0	43.4	29.6
Mineral mix	35.0	33.9	38.2
Vitamin mix	10.0	9.7	10.9
Choline bitartrate	2.5	2.4	2.7
Terbutylhydroquinona antioxidant	0.008	0.008	0.009
Water	72.5	67.4	65.2
Nutrients provided by 100g of the extract ¹:			
Protein	-	14.9	27.1
Lipid	-	2.3	11.14
Fiber	-	57.4	29.3
Polyphenols	-	0.7	3.5
Theobromine	-	1.2	1.8

¹ The cocoa fibre (CF) diet contained 50 g/kg of cocoa fibre extract whereas the C10 diet (10% cocoa) contained 100 g/kg of cocoa powder. REF: reference.

At the end of the 4 week nutritional intervention, all rats were again adapted to the treadmill for 1 week by increasing time and speed and then half of the rats were randomly selected to remain sedentary while the second half performed an exhausting running test in a treadmill (Figure 1). The final exhaustion test consisted in running with an initial speed of 18 m/min for 15 min, and from then on increasing 3 m/min every 2 min until exhaustion.

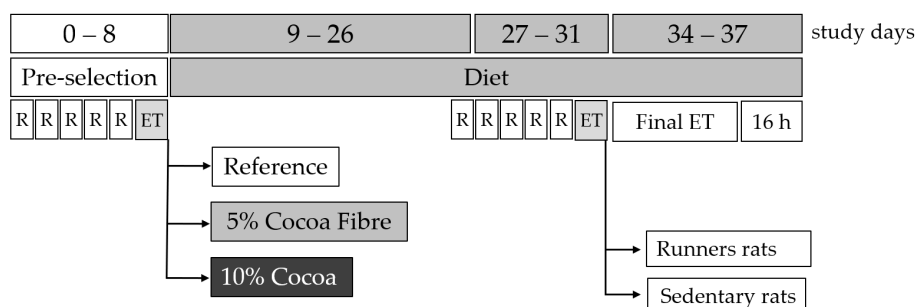


Figure 1. Experimental design. R = running session; ET = exhaustion test.

2.3. Sample Collection

In order to assess the oxidative stress and the Ig production, runner rats were euthanized 16 h after running the exhaustion test (ET). Likewise, sedentary rats were euthanized randomly over the same days. Animals were anaesthetized by intramuscular injection of ketamine (Merial Laboratories S.A., Barcelona, Spain) and xylazine (Bayer A.G., Leverkusen, Germany) ($90 \text{ mg} \times \text{Kg}^{-1}$ and $10 \text{ mg} \times \text{Kg}^{-1}$, respectively) and exsanguinated. Blood, peritoneal macrophages, submaxillary salivary glands (SMG), caecal content (CC) and the small intestine were collected.

SMG and CC were homogenised using a tissue homogeniser (Polytron, Kinematica, Lucerne, Switzerland and Pellet Pestle Cordless Motor, Kimble, Meiningen, Germany, respectively), as previously described [5], and kept at $-20 \text{ }^{\circ}\text{C}$ until IgA quantification.

The proximal part of the small intestine was used to obtain gut wash (GW), as previously described [6]. Briefly, the intestine was washed with cold PBS (pH 7.2) in order to remove faecal content, opened lengthwise, cut into small pieces, weighed, incubated for 10 min in a shaker at $37 \text{ }^{\circ}\text{C}$ ($55 \text{ shakings} \times \text{min}^{-1}$) and centrifuged (538 g , $4 \text{ }^{\circ}\text{C}$, 10 min). Then, supernatants were collected and stored at $-20 \text{ }^{\circ}\text{C}$ until IgA quantification.

2.4. Peritoneal Macrophage Isolation and ROS Production

Peritoneal macrophages were obtained as described previously in our laboratory [7]. Briefly, cells were collected after a 2 min massage with a 40 mL sterile PBS injected in the peritoneal cavity, centrifuged and resuspended with cold Roswell Park Memorial Institute (RPMI) medium without phenol red and supplemented with 1% heat-inactivated fetal bovine serum, 100 IU/mL streptomycin–penicillin, 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Madrid Spain). After cell counting using a Spincell hematology analyzer (MonLab Laboratories, Barcelona, Spain), macrophages were plated (10^4 cells/well) on a 96-well black plate (Sigma-Aldrich) and incubated overnight in order to assess the ROS production [7]. Then, cells were washed and incubated for 30 min with $20 \text{ }\mu\text{M}$ of reduced 2',7'-dichlorofluorescein diacetate probe ($\text{H}_2\text{DCF-DA}$, Invitrogen, Paisley, UK) in order to oxidise the $\text{H}_2\text{DCF-DA}$ to a fluorescent compound (2',7'-dichlorofluorescein) by means of the macrophage-derived ROS. The obtained fluorescence was measured at 0 and 120 min by the fluorimeter Modulus Microplate Multimode Reader (Turner BioSystems, CA, USA). ROS data are expressed as the percentage of increase between 0 and 120 min.

2.5. Immunoglobulin Quantification

The concentrations of IgG, IgM and IgA in serum, SMG or GW were quantified by a sandwich ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA), as previously described [8]. Absorbance was measured on a microplate photometer (Labsystem Multiskan, Helsinki, Finland) and data were interpolated according to the respective standard curve by Ascent v.2.6 software (Thermo Fisher Scientific, Barcelona, Spain). In order to normalize the Ig content in SMG, total protein concentration was measured using the Pierce-660 nm Protein Assay Reagent (Thermo Fisher Scientific).

3. Results and Discussion

3.1. ROS Production by Peritoneal Macrophages

The production of ROS from peritoneal macrophages obtained from sedentary and runner rats, fed either with the REF diet, CF diet and C10 diet, was quantified (Figure 2). The ROS production was increased in the acutely exercised rats from the REF group ($p < 0.05$ vs. REF-SED group), which was prevented by the cocoa intake, especially by means of its fibre content ($p < 0.05$, CF-RUN group vs. REF-RUN group).

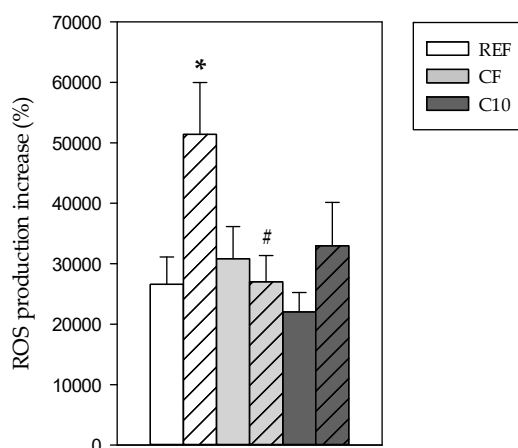


Figure 2. Reactive oxygen species (ROS) production by peritoneal macrophages at the end of the study (percentage of increase from 0 to 120 min). The sedentary groups are represented by bars without any pattern and the runner groups by a striped pattern. REF = reference diet; CF = 5% cocoa fibre enriched diet; C10 = 10% cocoa enriched diet. Data are expressed as mean \pm SEM ($n = 8$). * $p < 0.05$ vs. the sedentary group in the same dietary group; # $p < 0.05$ vs. the same exercise condition in the REF diet.

3.2. Immunoglobulin Production

The serum contents of IgG, IgM and IgA 16 h after performing the running exhaustion test were quantified (Figure 3).

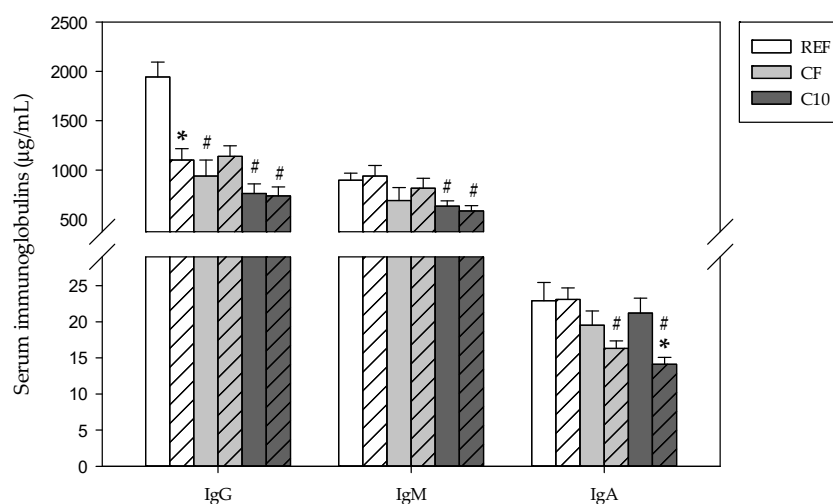


Figure 3. Immunoglobulin concentrations (µg/mL) in serum at the end of the study. The sedentary groups are represented by bars without any pattern and the runner groups by a striped pattern. REF = reference diet; CF = 5% cocoa fibre enriched diet; C10 = 10% cocoa enriched diet. Data are expressed as mean \pm SEM ($n = 8$). * $p < 0.05$ vs. the sedentary group in the same dietary group; # $p < 0.05$ vs. the same exercise condition in the REF diet.

Acute exercise decreased the serum IgG concentration ($p < 0.05$ vs. REF-SED group), nevertheless, these lower levels were also found in the CF and C10 dietary groups, even in the sedentary animals. Neither IgM nor IgA were modified by acute exercise, however the C10 diet decreased the IgM content in both sedentary and runner rats ($p < 0.05$ vs. REF-SED and REF-RUN, respectively) and both CF and C10 diets decreased the IgA concentration only in exercised rats ($p < 0.05$ vs. REF-RUN; $p < 0.05$ between C10-SED and C10-RUN).

The salivary gland IgM and IgA content and the IgA concentration in GW were also assessed (Figure 4). Acute exercise decreased the salivary gland IgM content in the REF group ($p < 0.05$ vs. REF-SED group), whereas the CF diet tended to attenuate this effect ($p = 0.064$, between the CF-RUN group and the REF-RUN group), the C10 group showed much lower levels ($p < 0.05$, C10-RUN group vs. REF-RUN group and CF-RUN group), even in sedentary animals ($p < 0.05$, C10-SED group vs. REF-SED group and CF-SED group). Moreover, both CF and C10 diets decreased the salivary gland IgA content, evidencing a higher impact in CF runner animals than in the sedentary ones ($p < 0.05$, between CF-RUN and CF-SED). Regarding the intestinal IgA, C10 runner rats showed lower levels than the sedentary ones and the REF runner rats ($p < 0.05$).

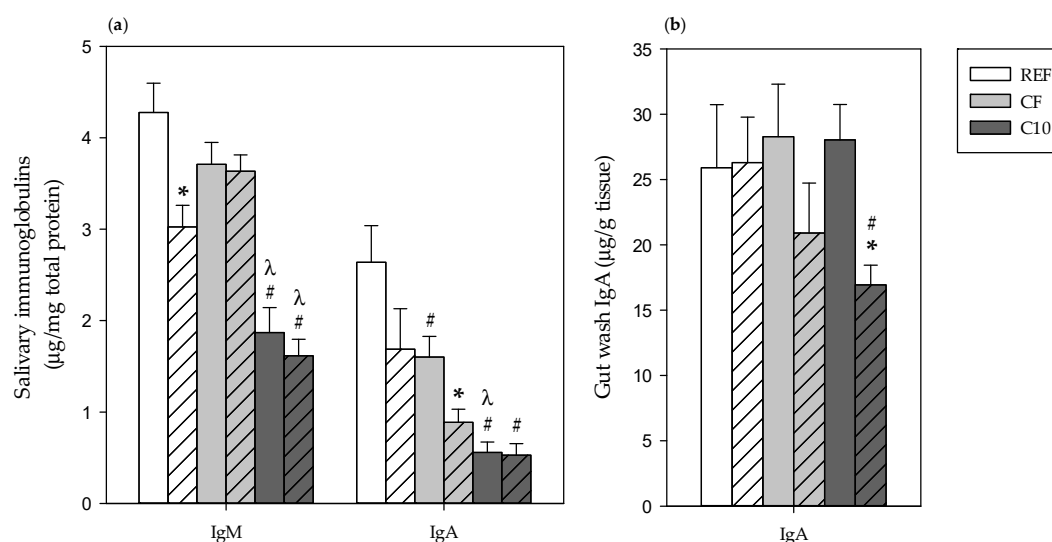


Figure 4. Submaxillary salivary gland IgM and IgA (a), and gut wash IgA (b) concentration at the end of the study. The sedentary groups are represented by bars without any pattern and the runner groups by a striped pattern. REF = reference diet; CF = 5% cocoa fibre enriched diet; C10 = 10% cocoa enriched diet. Data are expressed as mean \pm SEM ($n = 8$). * $p < 0.05$ vs. the sedentary group in the same dietary group; # < 0.05 vs. the same exercise condition in the REF diet; $\lambda < 0.05$ vs. the same exercise condition in the CF diet.

In summary, acute exercise increased ROS production and decreased the serum IgG concentration and the salivary gland IgM content. The cocoa fibre-enriched diet prevented both the higher production of ROS and the reduction in salivary IgM induced by exercise, although it decreased the IgA content in serum and salivary glands. The cocoa diet also altered the Ig profiles, evidencing a higher impact in runner animals than in the sedentary ones.

4. Conclusions

Overall, cocoa, by means of its fibre content, can partially prevent the alterations in ROS, and Ig production induced by a single session of intensive running exercise.

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Conflicts of Interest: The authors declare no conflict of interest.

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