Comparison of immunomodulatory proprieties and antioxidant effect between chicken protein, casein and gluten consumption in mice

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ABSTRACT

This study was designed to evaluate the immunomodulatory proprieties and antioxidant effect of chicken protein by comparing with gluten and casein consumption. 18 Six-week-old female Balb/c mice were randomly separated into 3 groups. Without acclimation, mice were fed ad libitum for 8 weeks with AIN-93G (without protein) supplemented with casein or gluten or chicken protein (20% of the total amount) for casein, gluten and meat group respectively. Flow cytometric analysis was used to measure the cell surface expression of CD11b as macrophage marker, and CD11c+ cells in the spleen. In addition, for distinguishing the phenotype of macrophage, ELISA was performed to measure the interferon-ɣ and interleukin-10 in the supernatant of splenocytes cultured with 10µg/mL of concanavalin A. Oxidative stress was evaluated by measuring the total reactive oxygen species generation in liver. For this purpose, the probe, 2', 7'- dichloro-dihydrofluorescein-diacetate was used, and the result was expressed as fluorescence intensity per mg of protein in the liver homogenate. Regarding the results, splenocytes isolated from mice for each group contained high rate of macrophage, 5.71±0.41%, 5.95±0.47% and 5.24±0.63% for casein, gluten and meat group respectively. Yet, the level of interferon-ɣ and interleukin-10 in the supernatant of splenocytes cultured with concanavalin A for 96h showed no significant differences. In addition, the variation intra-group was a bit high. In fact, interferon -ɣ production was ranged from 30.57 to 162.95 pg/mL, 0 to 122.95 pg/mL and 16.29 to 188.67pg/mL for casein, gluten and meat group respectively. Interleukin-10 production was varied from 7.75 to 185.25pg/mL, 0 to 75.25pg/mL and 0 to 272.75pg/mL for casein, gluten and meat group respectively. Concerning reactive oxygen species generation, no significant difference was also found. However, the trend showed that meat group produced less reactive oxygen species than the 2 other dietary groups. In fact, it was ranged from 0.49±0.03 to 0.96±0.06, 0.68±0.16 to 1.12±0.22 and 0.49±0.08 to 0.74±0.11 fluorescence intensity/mg of protein for casein, gluten and meat group respectively after 40 and 80min of incubation of liver homoginate with DCFDA. Taken together, despite the high rate of macrophage, it was difficult to draw any conclusion about the dominant phenotype in each group. Concerning the antioxidant effect, the trend showed that meat group produces less reactive oxygen species than the other 2 dietary groups.

Keywords: *Chicken protein, immunomodulatory, macrophage, oxidative stress, mice*

1. INTRODUCTION

Food of animal origin including meat is required to maintain the health of human body [1]. Because, meat is specifically valuable as a source of many nutrients with high biological value such as protein, vitamin B (B12), omega-3 fatty acid, highly bioavailable iron [2]. It was thought that consumption of meat, particularly rich in unsaturated fats, is the factor responsible for the increase in brain size over the last 4.5 million years [3 - 4]. Apart from being a good source of potential nutrients, meat such as chicken meat contains also many

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bioactive compounds which can protect and maintain the human body healthy. In fact, chicken meat contains some antioxidant compounds namely anserine (β-alanyl-N-methylhistidine) and carnosine (β-alanyl L-histidine) which are capable of inhibiting lipid oxidation [5 - 6]. In addition, it was reported that hydrolyzed chicken extract can inhibit Angiotensin -I converting enzyme (ACE) which is an enzyme playing a leading part in the increase of blood pressure [7]. Moreover, Chicken extract can reduce pro-inflammatory cytokine production in KOR-ApoEshl mice model. Besides, Sim et al. reported that chicken meat had a hypoglycemic action in type-2 diabetic KKAy mice and GK rats [8].

Unfortunately, despite the presence of a number of potential and protective bioactive compounds, too much consumption of meat was thought to contribute to the progression of chronic diseases such as cardiovascular diseases, cancer, type 2 diabetes, hypertension, and high blood lipid [9, 10].

As discussed above, meat consumption is necessary for human health and well-being by consuming moderately. For this purpose, this study was designed to evaluate the immunomodulatory proprieties and antioxidant effect of chicken protein consumption by comparing with gluten and casein consumption in mice.

2. MATERIAL AND METHODS

2.1. Reagent

Hanks' Balanced Salt Solutions (HBSS), RPMI-1640 medium, penicillin and streptomycin were purchased from Life Technologies (Foster City, CA, USA). Fetal bovine serum (FBS) was obtained from ICN Biomedicals (Osaka, Japan). ELISA kit was purchased from DuoSet (R&D Systems). Chicken protein was kindly provided by R&D Center, Nippon Meat Packers Inc. (Tsukuba, Ibaraki, Japan). Casein and Gluten were purchased from Wako Pure Chemical industries Ltd. (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan) respectively. Finally, 2', 7' Dichloro-dihydrofluorescein diacetate (DCFDA) was purchased from Sigma Aldrich Japan.

2.2. Experimental animal and diet

Six-week-old female Balb/c mice (n=18) were obtained from Charles River (Kanagawa, Japan). Without acclimation, mice were randomly divided into 3 groups. The experiment was carried out according to the experimental protocol approved by the Animal Care Committee, Graduate School of Biosphere Science, Hiroshima University. Mice were housed in cage with a 12-h light: dark cycle (light 8:00 am-8:00pm) in an air conditioner room (24±1°C and 60% humidity). They were fed ad libitum for 8 weeks with AIN-93G (without protein) supplemented with casein or gluten or chicken protein (20% of the total amount) for casein, gluten and meat group respectively. The animal food was cleaned up everyday in order to discard the excreta, and the drinking water was changed every two days. The body weight was measured 3 times a week. At the end of the feeding period, all mice were sacrificed by performing a cervical dislocation. Then, blood, tongue and organs such as spleen, liver and brain were collected, snap-frozen in nitrogen liquid and stored at – 80°C, except spleen, for further experiments.

2.3. Murine splenocytes preparation and stimulation

The preparation of splenocytes was performed as previously described but slightly modified (http://www.thelabrat.com/protocols/splenocyteprep.shtml) [11].

2.4. Flow cytometric analysis of macrophage and CD11c+cells in spleen

Flow cytometry was used to measure the cell surface expression of CD11b as macrophage marker in general, and CD11c as dendritic cells marker but also considered as classically M1 macrophage marker [12].

2.5. Measurement of cytokine level

The level of interferon (IFN)-ɣ and interleukin (IL)-10 were measured in the supernatant of splenocytes cultured with Con A (for 24h and 96h incubation). Sandwich ELISA (DuoSet. R&D Systems) was used according to the manufacturer's instruction [12 - 13].

2.6. Total ROS generation assay

First of all, liver homogenate was prepared according to the method described previously but slightly modified [13]. Concerning the ROS generation assay, it was performed as the method described previously but slightly modified [14].

2.7. Statistical analysis

Data are presented as mean ± SEM, and analyzed using one-way ANOVA. The statistical significance was set at $p<0.05$.

3. RESULTS

3.1. Increase in body weight

Each mouse gained weight during the period feeding. However, it appears that this increase in BW varied slightly among the mice belonging to each group. It was ranged from 4.58 to 10.47g, 2.56 to 10.06g and 3.29 to 8.45g for casein, gluten and meat group respectively. No significant difference was found among any of the 3 groups. Figure 1 shows the increase in body weight during the feeding period and table 1 presents the gain in body weight at the end of the feeding period for each mouse. Appendix 1 presents the preliminary results (feeding period 4 weeks) concerning the increase in body weight.

Figure 1. Increase in body weight for each dietary group during the feeding period

Table 1. Gain in body weight for each mouse at the end of the feeding period

	Casein (g)	Gluten (g)	Meat (g)
Mouse 1	10.47	3.70	5.97
Mouse 2	8.82	10.06	4.33
Mouse 3	4.58	5.08	8.45
Mouse 4	5.39	2.56	3.29
Mouse 5	4.78	3.47	4.84
Mouse 6	7.64	9.09	5.33
Mean ± SE	**6.95±0.99**	**5.66±1.29**	**5.37±0.72**

3.2. Splenic macrophage and CD11c+ cells

During the preliminary study, four types of immune cells were measured in the spleen, such as T cells, B cells, dendritic cells, and macrophage (see appendix 3). The trend showed that the percentage of splenic macrophage was a bit high in gluten and meat group. In order to follow this hypothesis, during this study, only splenic macrophage, and CD11c+ cells were taken into account. The rate of splenic macrophage was high for each group. However, no significant difference was found. Figure 2 shows the flow cytometric result, and table 2 presents the detail about it. Regarding CD11c+ splenocyte, no significant difference also was detected among any of the dietary group. Figure 3 and table 3 show the result about the rate of CD11c+ splenocytes.

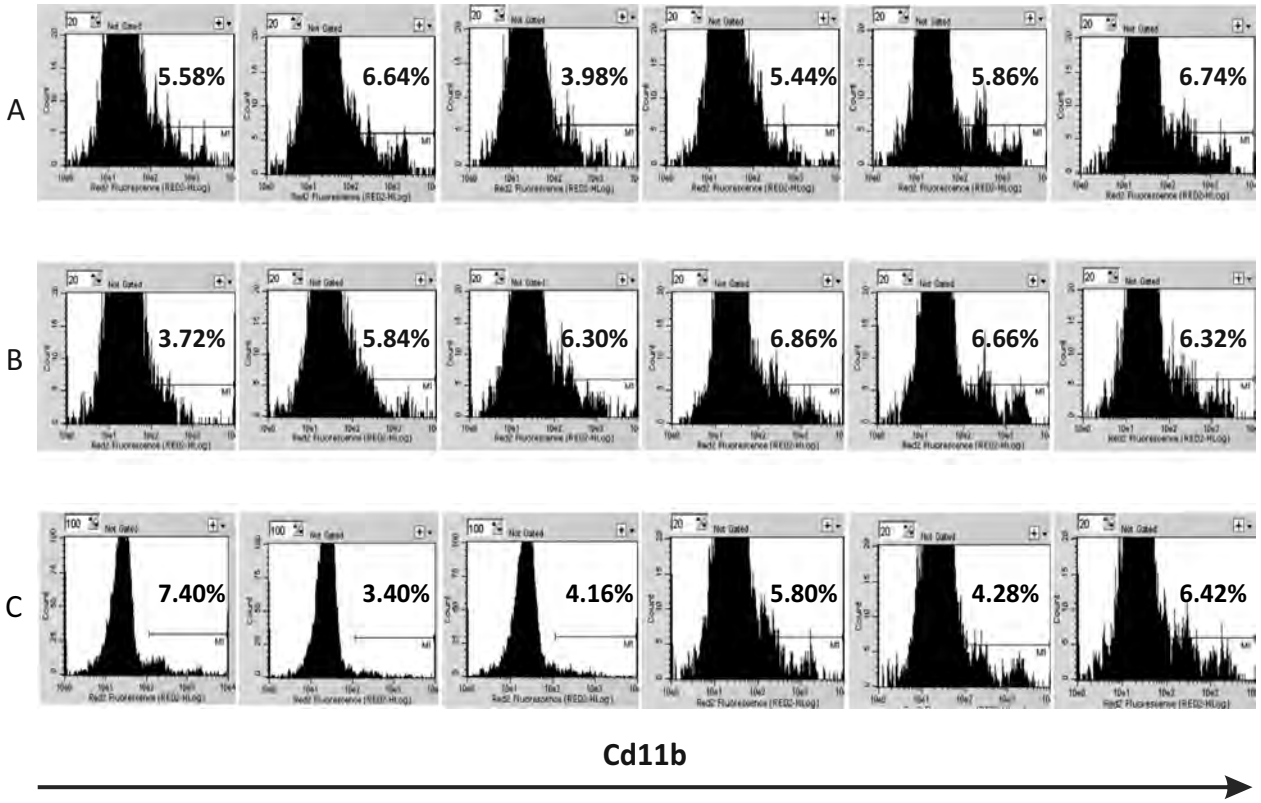

Figure 2. flow cytometric analysis for splenic macrophage by the expression of CD11b marker, (A) casein group, (B) gluten group and (C) meat group

Table 2. Rate of splenic macrophage for each mouse

	Casein (%)	Gluten (%)	Meat (%)
Mouse 1	5.58	3.72	7.40
Mouse 2	6.64	5.84	3.40
Mouse 3	3.98	6.30	4.16
Mouse 4	5.44	6.86	5.80
Mouse 5	5.86	6.66	4.28
Mouse 6	6.74	6.32	6.42
Mean	5.71±0.41	5.95±0.47	5.24±0.63

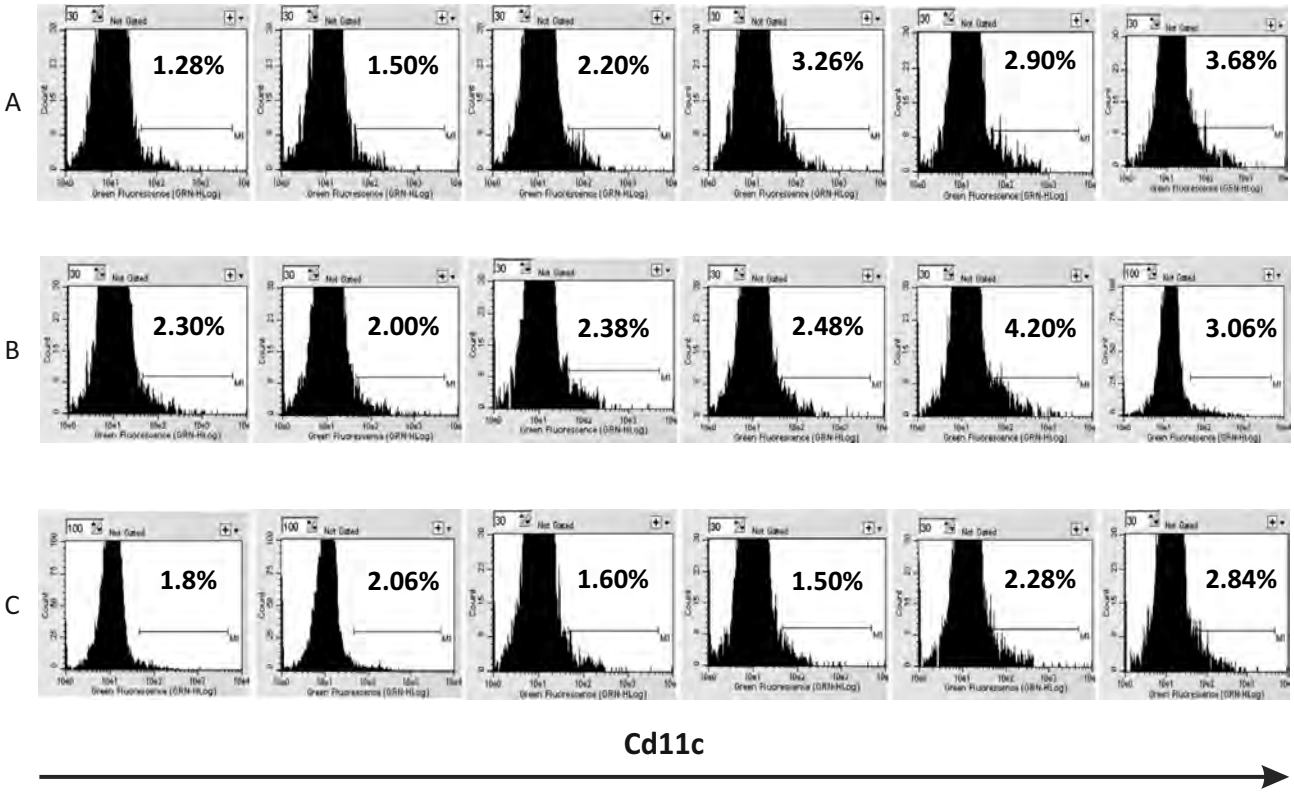

Figure 3. Flow cytometric analysis for CD11c + spleen cells, (A) casein group, (B) gluten group, (C) meat group

Table 3. Rate of CD11c+ splenocyte for each mouse

	Casein (%)	Gluten (%)	Meat (%)
Mouse 1	1.28	2.30	1.80
Mouse 2	1.50	2.00	2.06
Mouse 3	2.20	2.38	1.60
Mouse 4	3.26	2.48	1.50
Mouse 5	2.90	4.20	2.28
Mouse 6	3.68	3.06	2.84
Mean	2.47±0.40	2.74±0.33	2.01±0.20

It was reported that macrophage consists of at least two different phenotypes, classically activated M1 macrophage and alternatively activated M2 macrophage. M1 macrophage produces proinflammatory cytokine such as IFN-ɣ, Tumor necrosis factor (TNF)-α, IL-6, monocyte chemoattractant protein (MCP)-1, thus contributing to the induction of chronic diseases for example insulin resistance [15]. Whereas, M2 macrophage was involved in the resolution of inflammation, by secreting IL-10 and TGF-β, in repair and in wound remodeling of tissue via angiogenesis [16]. According to this information, and in order to define the dominant phenotype for each dietary group, the level of IFN- ɣ and IL-10 production were measured.

3.3. Cytokine productivity

Interferon (IFN)-ɣ and IL-10 were not detected in any of the supernatant of splenocyte

cultured with Con A for 24h incubation. Regarding 96h incubation, no significant differences were found among any of the 3 groups. Besides, the variation intra-group was a bit high. The production of IFN -ɣ was ranged from 30.57 to 162.95 pg/mL, 0 to 122.95 pg/mL and 16.29 to 188.67pg/mL for casein, gluten and meat group respectively. Regarding IL-10, the rate of production was varied from 7.75 to 185.25pg/mL, 0 to 75.25pg/mL and 0 to 272.75pg/mL for casein, gluten and meat group respectively. It was noticed that IFN-ɣ and IL-10 were not detected in the supernatant of splenocytes from mice 2 and 4. Table 4 shows more details about these data. Appendix 3 presents the preliminary results.

Table 4. INF-ɣ and IL-10 production in the supernatant of splenocytes culture with Con A for 96h

	Casein		Gluten		Meat	
	IL-10 (pg/mL)	IFN-ɣ (pg/mL)	IL-10 (pg/mL)	IFN-ɣ (pg/mL)	IL-10 (pg/mL)	IFN-ɣ (pg/mL)
Mouse 1	47.75	112.48	75.25	89.62	0.00	88.67
Mouse 2	10.25	162.95	0.00	0.00	92.75	33.43
Mouse 3	30.25	30.57	22.75	72.48	272.75	16.29
Mouse 4	7.75	62.00	0.00	0.00	0.00	145.81
Mouse 5	115.25	140.10	0.25	104.86	80.25	130.57
Mouse 6	185.25	109.62	15.25	122.95	0.00	188.67
Mean ± SE	66.08±28.73	102.95±20.03	18.92±11.92	64.98±21.65	74.29±43.33	100.57±27.36

3.4. ROS generation

No significant difference was found concerning the generation of ROS after 40min and 80min incubation of the liver homogenate with DCFDA. However, the trend shows that meat group produces less than the other dietary groups. In fact, it was ranged from 0.49±0.03 to 0.96±0.06, 0.68±0.16 to 1.12±0.22 and 0.49±0.08 to 0.74±0.11 fluorescence intensity/mg of protein for casein, gluten and meat group respectively after 40 and 80min of incubation with DCFDA. Figures 4 show respectively the ROS detection for each dietary group after 40min and 80min of incubation of liver homogenate.

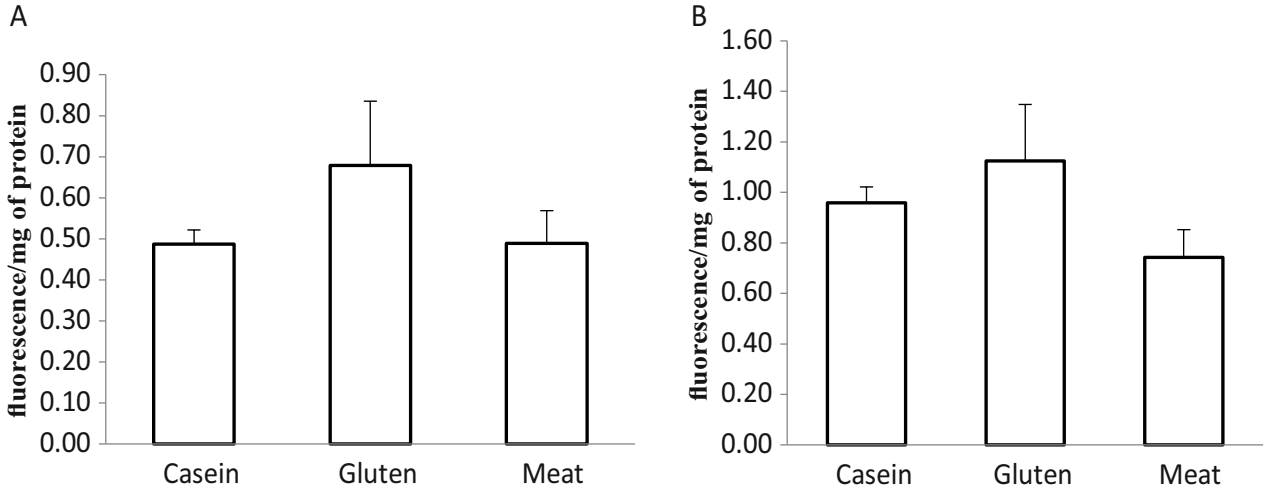

Figure 4. ROS detection for each dietary group after 40min (A) and 80 (B) incubation of liver homogenate with DCFDA

4. DISCUSSION

During the preliminary experiment (feeding period 4 weeks), the increase in body weight for meat group was progressive. However, during this second experiment, it was slowed down during the second month of consumption (feeding period 8 weeks). The reason was not yet clear, but by hypothesis, it may be related to the duration of feeding period, because it was reported that long term consumption, ranging from 2 weeks to one year *ad libitum* of high protein diet can cause a weight loss [17]. In addition, Paddon-Jones *et al*. reported that protein tends to be more satiating than the other macro-nutrients, both at the level of a single eating occasion and over days and weeks [18].

Concerning the increase in splenic macrophage in meat group, the cause was not yet clear, because even in the other dietary group, it was high. However, it was reported that there is a relationship between food intake and increase in splenic macrophages [19 - 20]. Concerning the IFN-ɣ and IL-10 production, the variation intra-group was so high, for example IL-10 and IFN-ɣ were not detected in the supernatant of splenocytes from some mice cultured with Con A. Consequently, it was difficult to describe the dominant phenotype of splenic macrophage for each group. For further experiment, the proceeding may be reviewed and considered. In fact, Petursdottir and Hardardottir reported that for IL-10 production purpose, Con A stimulates more T cells to secrete IL-10 (and TNF-α) by binding to CD3 molecule or perhaps also via CD2 [20]. Whereas, IL-10 (and TNF-α) measured following stimulation with lipopolysaccharide (LPS) was most likely secreted by macrophage as LPS binds to CD14 expressed on monocyte and macrophage. Concerning the generation of ROS in liver, no significant difference was found but the trend show that in meat group produces less ROS than the other groups. In fact, it was reported that meat contains some protein –related bioactive compound which can prevent oxidative damage. This action was attributed to neutralization and reduced release of free radicals by inhibiting lipid oxidation [5 - 6]. For further experiment, ROS generation will be taken again into account, and the proceeding also will be reviewed.

5. CONCLUSION AND PERSPECTIVE

Taken together, the number of splenic macrophage in meat group was a bit high. However, the reason was not yet clear, because even in the other dietary groups, it was high. There is no significant difference among any of the 3 dietary groups. Concerning the predominant phenotype of macrophage, it was a bit difficult to draw any conclusion, because the variation intra-group on IL-10 and IFN-ɣ production was so high. Concerning the antioxidant effect of meat consumption in liver, no significant difference was also found. But, the trend showed that meat group produces less ROS than the other groups.

For further experiments, the dominant phenotype of macrophage and ROS generation will be again taken into account. In fact, there was a trend, but the difference was not statistically significant. In addition, each data from each mouse is not uniform. For this purpose, the proceeding will be reviewed. Concerning macrophage phenotype particularly, it is difficult to draw any conclusion. So if it is necessary, the indicators used, from which we can conclude concerning the dominant phenotype, will be considered. On the other hand, the effect of meat consumption on taste and olfactory acuity will be evaluated.

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So sánh các đặc tính điều hòa miễn dịch và tác dụng oxy hóa của việc hấp thụ protein, casein và gluten ở chuột

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TÓM TẮT

Nghiên cứu này được thiết kế để đánh giá các đặc tính điều hòa miễn dịch và tác dụng chống oxy hóa của protein gà bằng cách so sánh với mức tiêu thụ gluten và casein. 18 chuột Balb/c cái 6 tuần tuổi được chia ngẫu nhiên thành 3 nhóm. Những con chuột được cho ăn tự do trong 8 tuần với AIN-93G (không có protein) được bổ sung casein hoặc gluten hoặc protein gà (20% tổng lượng) cho nhóm casein, gluten và thịt tương ứng. Phân tích tế bào được sử dụng để đo biểu hiện bề mặt tế bào của CD11b dưới dạng chất đánh dấu đại thực bào và các tế bào CD11c+ trong lá lách. Ngoài ra, để phân biệt kiểu hình của đại thực bào, ELISA đã được thực hiện để đo IFN-ɣ và IL-10 trong dịch nổi của các tế bào lách được nuôi cấy với 10µg/mL concanavalin A. Sự căng thẳng oxy hóa được đánh giá bằng cách đo tổng số loại oxy phản ứng được tạo ra trong gan. Với mục đích này, mẫu dò, 2', 7'- DCFDA đã được sử dụng, và kết quả được biểu thị bằng cường độ huỳnh quang trên mỗi mg protein trong dịch gan đồng nhất. Các tế bào lách được phân lập từ chuột cho mỗi nhóm chứa tỷ lệ đại thực bào cao, lần lượt là 5,71 ± 0,41%, 5,95 ± 0,47% và 5,24 ± 0,63% đối với nhóm casein, gluten và thịt. Tuy nhiên, mức độ IFN-ɣ và IL-10 trong dịch nổi của tế bào lách được nuôi cấy bằng concanavalin A trong 96 giờ cho thấy không có sự khác biệt đáng kể. Ngoài ra, sự khác biệt trong nhóm là có thay đổi. Trên thực tế, sản xuất IFN-ɣ dao động từ 30,57 đến 162,95 pg/mL, 0 đến 122,95 pg/mL và 16,29 đến 188,67 pg/mL đối với nhóm casein, gluten và thịt tương ứng. Sản xuất IL-10 thay đổi từ 7,75 đến 185,25pg/mL, 0 đến 75,25pg/mL và 0 đến 272,75pg/mL đối với casein, gluten và nhóm thịt tương ứng. Liên quan đến việc tạo ra các loại oxy phản ứng, cũng không có sự khác biệt đáng kể nào được tìm thấy. Tuy nhiên, xu hướng cho thấy nhóm thịt tạo ra ít oxy phản ứng hơn so với 2 nhóm ăn kiêng khác. Trên thực tế, nó nằm trong khoảng từ 0,49 ± 0,03 đến 0,96 ± 0,06, 0,68 ± 0,16 đến 1,12 ± 0,22 và 0,49 ± 0,08 đến 0,74 ± 0,11 cường độ huỳnh quang/mg protein đối với nhóm casein, gluten và thịt tương ứng sau 40 và

80 phút ủ của gan homoginate với DCFDA. Tổng hợp lại, mặc dù tỷ lệ đại thực bào cao, rất khó để đưa ra bất kỳ kết luận nào về kiểu hình chiếm ưu thế trong mỗi nhóm. Liên quan đến tác dụng chống oxy hóa, xu hướng cho thấy nhóm thịt tạo ra các loại oxy phản ứng ít hơn so với 2 nhóm ăn kiêng còn lại.

Từ khóa: *Protein, điều hòa miễn dịch, đại thực bào, stress oxy hóa, chuột*

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