

1 **Oral glucosylceramide reduces 2,4-dinitrofluorobenzene induced inflammatory response**
2 **in mice by reducing TNF-alpha levels and leukocyte infiltration**

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10 **Running title:** Anti-inflammatory Property of Orally Administered Glucosylceramide

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1 **Abstract**

2 Sphingolipids are constituents of cellular membranes and play important roles as second
3 messengers mediating cell functions. As significant components in foods, sphingolipids have
4 been proven to be critical for human health. Moreover, diverse metabolic intermediates of
5 sphingolipids are known to play key roles both in proinflammatory and in anti-inflammatory
6 effects. However, the effect of dietary sphingolipids on inflammation is a complicated field that
7 needs to be further assessed. Our study evaluated the effects of orally administered maize
8 glucosylceramide (GluCer), one of the most conventional dietary sphingolipids, on inflammation
9 using the 2, 4-dinitro-1-fluorobenzene (DNFB)-treated BALB/c murine model. Oral
10 administration of GluCer inhibited ear swelling and leukocyte infiltration to the inflammatory
11 site, suggesting that dietary GluCer has anti-inflammatory properties. ELISA analyses revealed
12 that oral administration of GluCer for 6 days was not modified the Th1/Th2 balance, but
13 significantly down-regulated the activation of TNF- α at the inflammatory site. Based on these
14 results, the down-regulation of TNF- α by dietary GluCer may suppress vascular permeability and
15 reduce the migration of inflammatory cells. Our findings increase understanding of the actions
16 dietary sphingolipids on the balance of the immune response.

17

1 **Keywords**

2 Sphingolipids, Dietary supplements, glucosylceramide, Anti-inflammatory agents, DNFB,
3 BALB/c mice, TNF- α , immune response

4

5

6 **Abbreviations**

7 DNFB 2, 4-dinitro-1-fluorobenzene

8 GluCer glucosylceramide

9 IFN- γ interferon-gamma

10 IgE immunoglobulin E

11 IL-1 β interleukin-1beta

12 IL-4 interleukin-4

13 IL-6 interleukin-6

14 NF- κ B receptor activator of nuclear factor-kappa B

15 Th1 T-helper 1

16 Th2 T-helper 2

17 TNF- α tumor necrosis factor-alpha

18

1 **Introduction**

2 Sphingolipids are commonly believed to protect the cell surface against harmful
3 environmental factors by forming the mechanically stable and chemically resistant outer leaflet
4 of the plasma membrane lipid bilayer [1-4]. Sphingolipids generate diverse metabolic
5 intermediates, notably ceramide, sphingosine, sphingosine-1-phosphate and
6 ceramide-1-phosphate, which serve as important mediators in the signaling cascades involved in
7 apoptosis, proliferation, and stress responses [5-8]. Although we have already demonstrated
8 that dietary sphingolipids are poorly absorbed by the intestine [9], sphingolipids that are
9 significant components of foods have gained considerable attention for their potential and
10 essential roles in human health [10-14]. It has been reported that dietary supplementation with
11 sphingolipids has diverse physiological effects, such as lowering plasma lipids [15], improving
12 skin barrier function [16], preventing melanin formation [17], contributing to central nervous
13 system myelination [18] as well as protecting the colon against inflammation [19-25]. However,
14 the functional, regulatory, and physiological significance of the immune regulating effects of
15 dietary sphingolipids is an appreciably complicated field that is not well understood.

16 One hypothesis of immune regulation involves the balance between T-helper 1 (Th1) and
17 T-helper 2 (Th2) cells, which direct different immune response pathways. Th1 cells drive the
18 "cellular immunity" pathway to fight viruses and other intracellular pathogens, eliminate cancer
19 cells, and stimulate delayed-type hypersensitivity skin reactions. Th2 cells are involved in
20 "humoral immunity" and up-regulate antibody production to fight extracellular organisms.
21 Either pathway can down-regulate the other. Disruption of the Th1/Th2 balance can cause
22 immunological diseases [26, 27]. Via the actions of sphingolipid degrading enzymes, such as
23 sphingomyelinase, glycosphingolipidases and ceramidase, dietary sphingolipids are hydrolyzed to
24 various kinds of metabolic intermediates which are critical for the activation and mediation of
25 various types of immune cells. Metabolites of sphingolipids initiate and maintain diverse
26 aspects of immune cell balance and functional responses by regulating cell migration and
27 inflammatory pathways [8, 20, 28-31]. For instance, sphingolipid hydrolysis products regulate

1 cyclooxygenase-2, interleukin 1 β (IL-1 β), interleukin 6 (IL-6), tumor necrosis factor α (TNF- α)
2 and nuclear factor kappa B (NF- κ B) via the sphingosine kinase 1/ sphingosine-1-phosphate and
3 ceramide kinase 1/ceramide-1-phosphate pathways, and thus cause the activation of mast cells,
4 control thymocyte maturation and regulate the balance of lymphocyte subpopulations [32-37].

5 The goal of this study was to evaluate the effects of orally administered glucosylceramide
6 (GluCer), one of the most important dietary sphingolipids, against DNFB-induced ear swelling in
7 the BALB/c murine model, to provide further understanding of how dietary sphingolipids act on
8 the balance between proinflammatory and anti-inflammatory responses.

9

10 **Materials and Methods**

11 **Maize GluCer preparation**

12 GluCer from maize was kindly donated by Nippon Flour Mills Co. Ltd. (Atsugi, Japan).
13 The purity of this GluCer was 96%, which was determined by HPLC equipped with an
14 evaporative light-scattering detector, as described previously [12].

15

16 **Animals**

17 Female BALB/c mice (6 weeks old, 15–20 g body weight) were purchased from Japan
18 SLC Inc. (Shizuoka, Japan). Animals were group-housed at 6 mice per cage, and were bred at
19 the Institute's animal facilities at 25 °C with a 12-hour light/dark cycle. Pure water and
20 AIN-93G diet (Oriental Yeast Co., LTD., Tokyo, Japan) were available *ad libitum*. All
21 experiments were performed according to the guidelines of Kyoto University for the use and care
22 of laboratory animals.

23

24 **Contact hypersensitivity induced by DNFB**

25 After a 2-week acclimatization period, allergic contact dermatitis was induced by DNFB in
26 BALB/c mice according to a previously published method with minor modifications [38].
27 Briefly, mice were sensitized on day 0 by application of 100 μ l 0.5% DNFB in acetone-soybean
28 oil (4:1, v/v) on their shaved dorsal skin. The mice were divided into control, low dose (5 mg)

1 and high dose (50 mg) groups (n=12 in each group). An identifying mark was made on the tail
2 of each mouse.

3 The maize GluCer was suspended in 0.5% carboxymethyl cellulose (CMC) (Nacalai
4 Tesque Co. Ltd., Kyoto, Japan) solution and was orally administered at 5 or 50 mg to each
5 mouse daily for six days. One hour after the final treatment, mice were challenged with 20 μ L
6 0.5% DNFB in acetone–soybean oil (4:1) on both ears. The thickness of the right ear of each
7 mouse was measured with a Dial Thickness Gauge (Mitutoyo Co., Kanagawa, Japan) at 0, 6 and
8 24 hours after the DNFB challenge. Ear swelling was calculated as the difference in thickness
9 before and after challenge [39].

10 Six hours (n=6) and 24 hours (n=6) after DNFB treatment, blood was collected and mice
11 were sacrificed under anesthesia. The right ear and spleen of each mouse was immediately
12 excised and frozen in liquid nitrogen, then stored at -80°C until use.

13

14 **Morphological analysis**

15 The left ear of each mouse was fixed in 10% neutral buffered formalin solution and was
16 then processed routinely into paraffin wax. Formalin fixed paraffin sections were stained with
17 hematoxylin and eosin (H&E) to observe morphological changes using a microscope (Keyence
18 Co., Osaka, Japan).

19

20 **Measurement of cytokine production and serum immunoglobulin E (IgE)**

21 Amounts of IFN- γ , interleukin-4 (IL-4), TNF- α and interleukin-10 (IL-10) in homogenates
22 of tissues were quantified using Murine IL-4 (Diaclone Research, Besancon, France), Murine
23 IFN- γ (Diaclone Research), Mouse TNF- α (Pierce Biotechnology Inc., Rockford, IL, USA),
24 and Murine IL-10 (Diaclone Research) ELISA kits, respectively, according to the manufacturer's
25 instructions. Levels of those cytokines in each supernatant were normalized to total protein
26 content, which was determined using a DC Protein assay kit (Bio-Rad Laboratories, Hercules,
27 CA, USA). Total serum IgE levels of DNFB-challenged mice were quantified using a Mouse
28 IgE ELISA kit (Immunology Consultants Laboratory, Newberg, Oregon, USA) according to the

1 manufacturer's instructions.

2

3 **Statistical analysis**

4 Data are reported as means \pm SD. Statistical analyses were performed using one-way
5 analysis of variance (ANOVA) with Fisher's PLSD method to identify levels of significance
6 between the groups.

7

8 **Results**

9 **GluCer suppresses DNFB-induced ear swelling of BALB/c mice**

10 After challenge with DNFB, typical allergic contact dermatitis was provoked in ears of
11 BALB/c mice, which was characterized by an initial increase of ear thickness and visible
12 congestion of blood vessels. Oral treatment with maize GluCer suppressed DNFB-induced
13 inflammatory symptom (redness and thickness) of ears. As shown in Fig. 1, a significant
14 depression of ear thickness was observed at 6 h in both low (5mg/day) and high dose (50mg/day)
15 GluCer treated groups ($p<0.05$). At 24 h, the average value of ear thickness was also reduced
16 by GluCer, but there were no statistical differences among the three groups, which may due to
17 large individual differences. The reduction of DNFB-induced ear swelling implies dietary
18 GluCer has anti-inflammatory property.

19

20 **GluCer inhibits inflammatory infiltrates in the ears of BALB/c mice**

21 Histological specimens of ears were prepared at 6 h and 24 h after topical application of
22 DNFB in BALB/c mice. In the control group, typical allergic contact dermatitis with congested
23 blood vessels and apparent edema could be observed by H&E staining. As shown in Fig. 2A
24 and Fig. 2D, microvascular dilations and dense leukocytes infiltrating the connective tissue,
25 which are characteristics of inflammatory reactions, were clearly observed in the control group.
26 At higher magnification, various kinds of migrated inflammatory cells could also be observed,
27 including fibrocytes, mononuclear cells, degranulated mast cells and other leukocytes. These

1 results confirm that DNFB induces severe inflammation in the ears of BALB/c mice and that a
2 variety of lymphocytes migrated out from blood vessels during this contact sensitivity procedure.
3 In both the low (5 mg/day) and the high (50 mg/day) dose GluCer-treated groups, microvascular
4 dilation and leukocytes in inflammatory infiltrates were inhibited at 6 h (Fig. 2 B&C) and 24 h
5 groups (Fig. 2 E&F). These results show that dietary GluCer inhibits microvascular dilation
6 and inflammatory infiltration of DNFB-induced BALB/c mice.

8 **GluCer inhibits inflammation by reducing TNF- α production in the ear**

9 To clarify the effect of GluCer on DNFB-induced inflammation, especially on Th1/Th2
10 balance, levels of IFN- γ as an indicator of Th1 cells and IL-4 as an indicator of Th2 cells were
11 measured by ELISA assay. IFN- γ and IL-4 levels of GluCer-treated group were not
12 significantly altered (Table 1). In other words, Th1/Th2 balance was not modified by oral
13 administration of GluCer for 6 days.

14 For further evaluating the effect of GluCer on DNFB-induced inflammation, IL-10 was
15 determined. In ear, IL-10 was significantly decreased at 6 h both low and high dose groups,
16 whereas this effect did not prolong to 24 h (Table 1). However, in spleen, IL-10 was increased
17 by GluCer treatment at 24 h, but not reached a statistical significance in 6 h because of the
18 relatively large individual differences (Table 1).

19 TNF- α as the most important proinflammatory cytokine and IgE as the most important
20 antibody in the serum were also measured. As shown in Fig. 3A, the TNF- α level in the ear
21 was significantly suppressed both in the low and high dose GluCer groups ($p < 0.05$). Moreover,
22 TNF- α level was also significantly down-regulated at 24 h by the effect of high dose GluCer (Fig.
23 3B). In contrast, IgE in the serum was almost at the same level among the control, low and
24 high GluCer dose groups (Fig. 3 C&D). The anti-inflammatory effect of dietary GluCer on
25 contact dermatitis in DNFB-induced BALB/c mice is via regulation of the level of TNF- α
26 secreted by inflammatory cells in the ear.

28 **Discussion**

1 The findings presented here indicate that dietary plant GluCer, one of the most important
2 and abundant sphingolipids in food [12], suppresses the DNFB-induced ear swelling of BALB/c
3 mice, and inhibits the microvascular dilation and inflammatory infiltration response via
4 down-regulating levels of TNF- α , but not modifying the balance of Th1/Th2. Meanwhile,
5 over-expressed IL-10 in inflammatory ear skin was suppressed by dietary GluCer. This
6 anti-inflammatory effect of dietary GluCer increases our understanding of biofunctional
7 sphingolipids.

8 Dietary GluCer is known to be hydrolyzed to ceramide, sphingosine and free fatty acids in
9 the intestinal lumen. In mucosal cells, exogenous free sphingosine and dihydrosphingosine are
10 rapidly absorbed and metabolized to palmitic acid [40]. A smaller portion of the sphingoid
11 bases is reincorporated into ceramide and more complex sphingolipids. Our recent findings
12 revealed that dietary GluCer originating from higher plants can be hydrolyzed in the intestine
13 and that the intact plant form of sphingoid bases is barely absorbed by the tissues [9,41]. Ono
14 *et al.* reported that dietary maize and yeast GluCer did not alter the sphingoid base composition
15 in the skin of NC mice [42]. We speculate that dietary maize GluCer accomplishes its
16 anti-inflammatory effect, not only by producing bio-active metabolic intermediates through the
17 sphingolipid metabolic pathways, but also via the activation of sphingolipid metabolic enzymes
18 that affect endogenous sphingolipids at the inflammatory site, because diverse metabolic
19 intermediates of sphingolipids, including ceramide, sphingosine, sphingosine-1-phosphate and
20 ceramide-1-phosphate are well known important and highly bioactive endogenous regulators,
21 which are involved in a complex metabolism network and play critical roles in inflammation
22 [43-46].

23 In the case of our study, dietary maize GluCer accomplishes its inhibition of inflammation
24 via the down-regulation of TNF- α level at the inflammatory site. TNF- α , produced by
25 mononuclear phagocytes and other inflammatory cells (neutrophils, lymphocytes, natural killer
26 cells and mast cells) or non-inflammatory cells (endothelial cells), is known to be one of the
27 most important inflammatory mediators [47,48]. TNF- α facilitates the formation of adhesion

1 molecules, vascular permeability and migration of leukocytes to sites of inflammation by
2 affecting endothelial cells [49-51]. Our results reveal that dietary maize GluCer down-regulates
3 levels of TNF- α in inflammatory ears. This down-regulation of TNF- α may affect endothelial
4 cells and inflammatory cells, and also can inhibit vascular permeability at the site of
5 inflammation. As a result, leukocyte migration is reduced. In the inflammatory cells, DNFB
6 activates NF- κ B by degrading the inhibitor of NF- κ B (I κ B) [52]. In addition, it has been
7 reported that sphingolipids down regulated TNF- α via inactivating of NF- κ B in
8 histamine-induced mouse skin tissues [53]. Thus, dietary GluCer seems to inhibit the DNFB
9 activated NF- κ B and down-modulate TNF- α expression in this study.

10 Moreover, IL-10 levels were increased in spleens but suppressed in ears by dietary GluCer.
11 This well known anti-inflammatory cytokine, IL-10, has been reported over-expressed during the
12 antigen-specific type of skin inflammation [54] and DNFB-challenged ear [55, 56]. IL-10 is
13 released by CD4⁺ T helper 2 (Th2) cell clones and a variety of other cells, including
14 keratinocytes, macrophages, B lymphocytes, and mast cells [56]. The suppressive effect of
15 dietary GluCer on IL-10 expression in DNFB-challenged ears might be caused by the inhibition
16 of leukocytes infiltrating to inflammatory site.

17 Ono *et al.* demonstrate that supplementation of 0.1% GluCer diet for 7-week prevented
18 atopic dermatitis-like symptoms in a mouse model by regulating the Th1/Th2 balance [42].
19 However, IFN- γ , IL-4 and IgE levels, the markers of Th1/Th2 balance, were not notably affected
20 by GluCer administration for 6 days in the present study. It appears that the period of treatment
21 is important for immunological response of dietary GluCer.

22 Furthermore, allergic inflammatory skin disease is associated with a loss of ceramide in
23 the extracellular lamellar membranes which causes an abnormal barrier function of the stratum
24 corneum [57]. Application of ceramide on diseased skin could significantly reduce allergic
25 inflammatory reactions by improving the severity score, stratum corneum cohesion and
26 hydration [58-60]. Dietary GluCer might also improve the stratum corneum cohesion and
27 hydration of ear skin to reduce the skin inflammation.

1 In summary, our data provide evidence that maize GluCer has anti-inflammatory effects on
2 the DNFB-induced inflammation of BALB/c mice. We confirmed that dietary GluCer inhibits
3 ear swelling and leukocyte infiltration. Furthermore, our results indicate that this effect is
4 accomplished mainly by down-regulating TNF- α , but does not significantly affect Th1/Th2
5 balance or IgE levels in the serum. We hypothesize that dietary GluCer accomplishes its
6 anti-inflammatory effect by down-regulating TNF- α to suppress vascular permeability. This
7 reduces the migration of inflammatory cells, affects endogenous sphingolipids through metabolic
8 pathways by activating sphingolipid-related enzymes, and moreover, by hydrolysis to ceramide
9 to improve skin barrier function at the dermatitis site. Our findings increase the comprehensive
10 understanding of the actions of dietary sphingolipids on the balance of immune responses.

11

12 **Acknowledgment**

13 This work was supported by the Program for Promotion of Basic and Applied Researches
14 for Innovations in Bio-oriented Industry (BRAINI).

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1 **Figure Legends**

2

3 Fig. 1 Effect of orally administered GluCer on DNFB-induced ear swelling in BALB/c mice.

4 The thickness of the left ear of each mouse was measured both before and after the DNFB
5 challenge. Ear swelling values are presented as the difference in thickness at 6 h (A) and at 24 h
6 (B): Ear swelling = ear thickness after challenge (6/24 h) – ear thickness before challenge (0 h).
7 Con, 6 days 0.5% CMC (vehicle) orally administered; Low, 6 days low dose (5 mg/day) maize
8 GluCer orally administered; High, 6 days high dose (50 mg/day) maize GluCer orally administered.
9 Values are means \pm SD, n = 6. Values with different superscript letters are significantly different
10 ($p < 0.05$).

11

12 Fig. 2 Histopathological analysis of orally administered maize GluCer on DNFB-induced ear
13 swelling in BALB/c mice.

14 Morphological changes in the left ear of 6 h and 24 h after DNFB-challenged BALB/c mice
15 were observed. Ear sections from control mice (A), low dose (5 mg/day) maize GluCer
16 administered mice (B) and high dose (50 mg/day) maize GluCer administered mice (C) were
17 stained with hematoxylin and eosin (H&E). Microvascular (asterisk marks) and leukocyte
18 (arrowheads) were pointed out in the histological sections. Sections are representatives of more
19 than five observations.

20

21 Fig. 3 TNF- α levels in the ears and IgE levels in the serum of DNFB- challenged BALB/c mice.

22 TNF- α (A,B) levels in the right ear homogenates and IgE levels in the serum (C,D) of
23 control, low dose (5 mg/day) and high dose (50 mg/day) maize GluCer administered mice were
24 measured both 6 (A,C) and 24 h (B,D) after the DNFB challenge. These data represent the
25 means \pm SD for groups of six mice. Values with different letters differ significantly ($p < 0.05$).

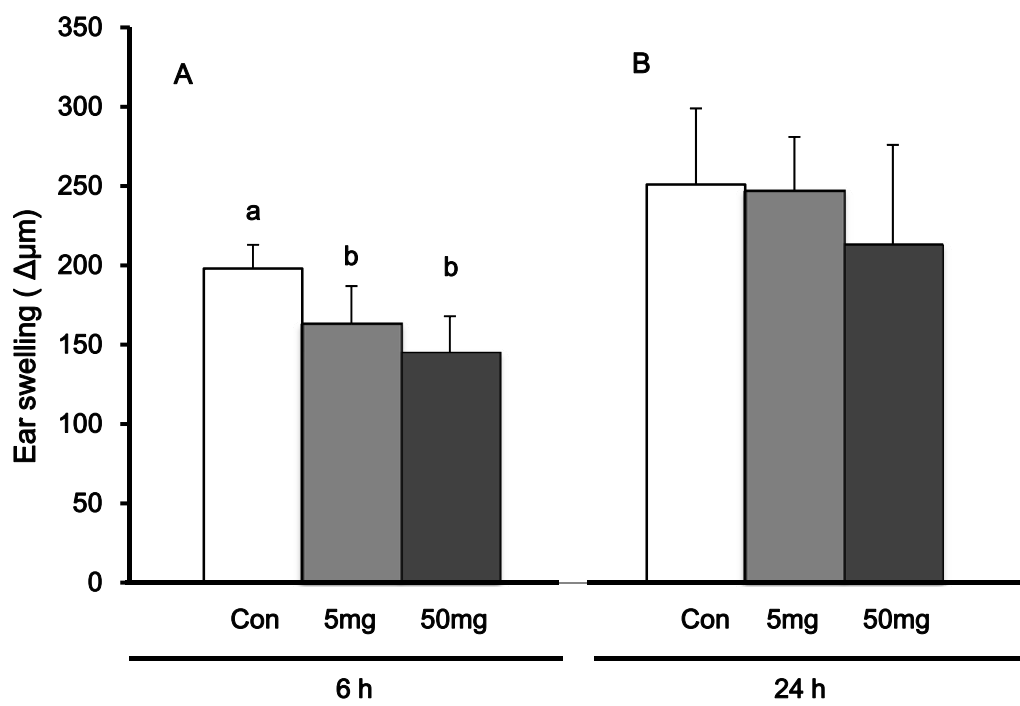


Fig. 1

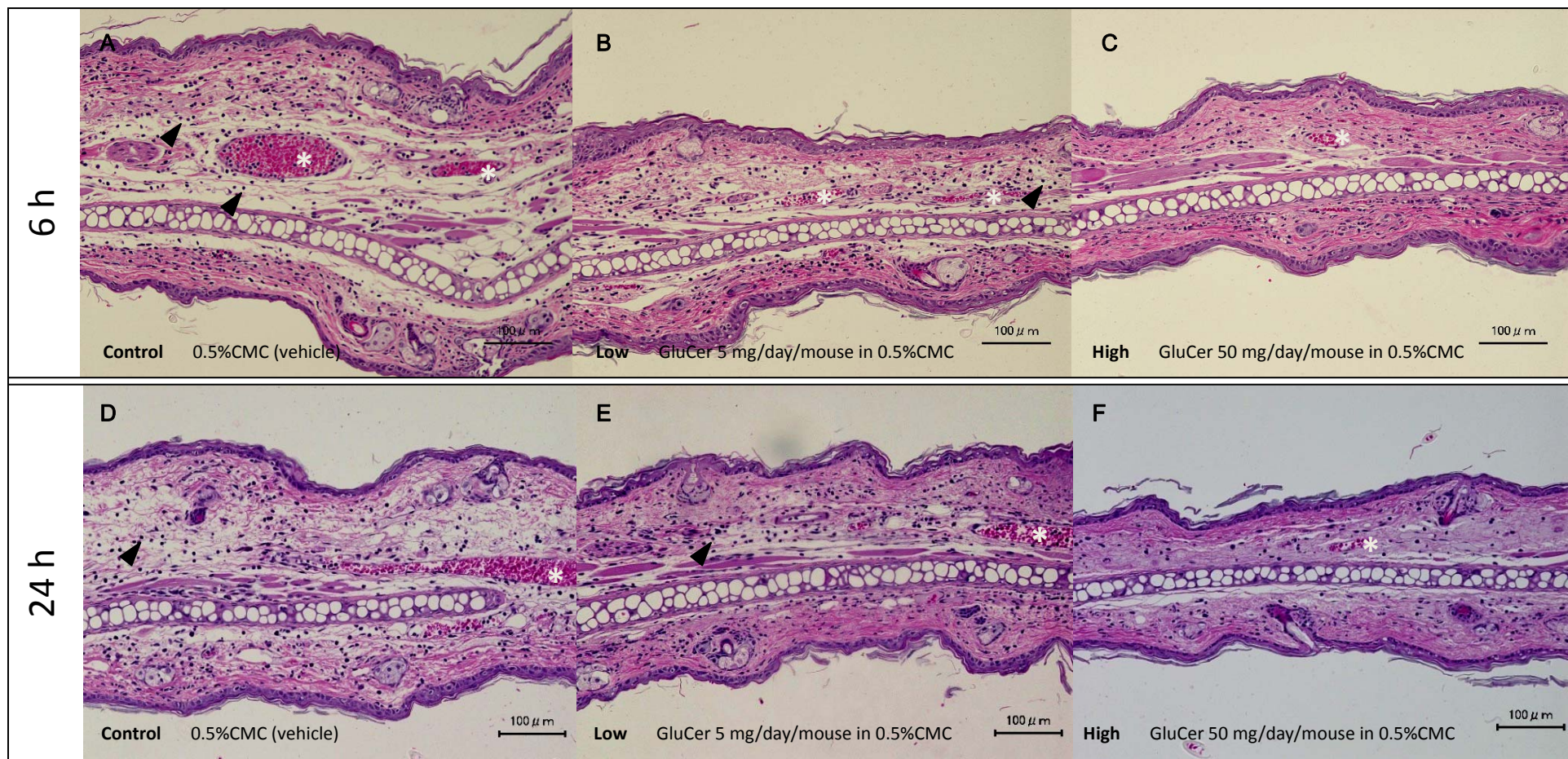


Fig. 2

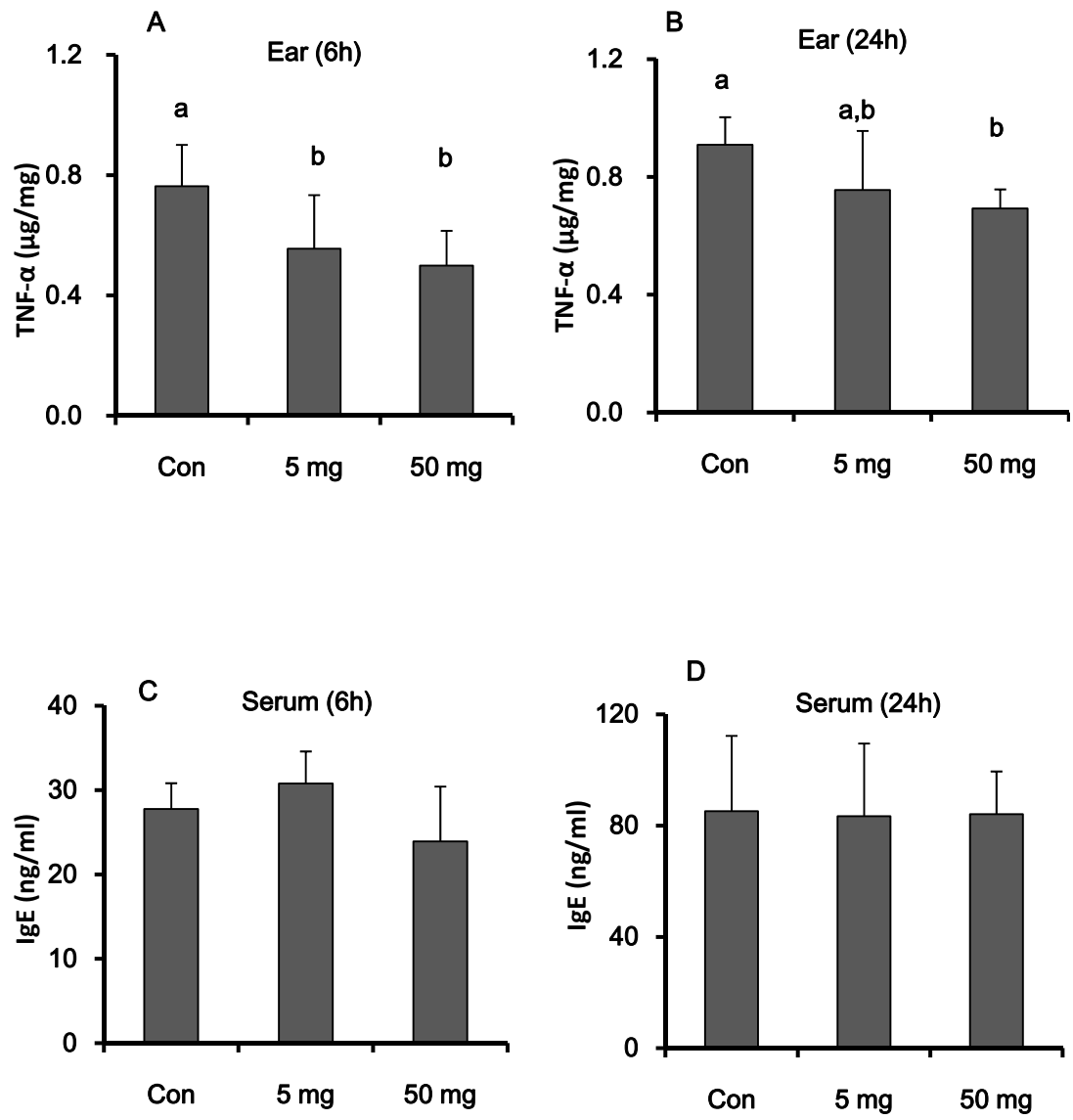


Fig. 3

Table 1. IFN- γ , IL-4 and IL-10 levels in ears and spleens of DNFB-challenged BALB/c mice.

Cytokines		Ear		Spleen	
		6 h	24 h	6 h	24 h
	mg/day				
IFN-γ	0	482.4 \pm 106.3	85.9 \pm 16.7	7.6 \pm 4.9	6.8 \pm 1.7
	5	362.0 \pm 89.4	89.1 \pm 18.3	7.5 \pm 4.1	8.5 \pm 2.7
	50	373.2 \pm 107.1	92.1 \pm 13.1	5.9 \pm 2.2	6.0 \pm 2.9
IL-4	0	25.3 \pm 4.4	10.3 \pm 2.5	2.8 \pm 0.9	1.9 \pm 0.4
	5	30.6 \pm 6.5	10.7 \pm 2.3	4.0 \pm 1.0	1.8 \pm 0.2
	50	29.8 \pm 8.4	9.0 \pm 2.1	3.2 \pm 0.7	1.5 \pm 0.2
IL-10	0	3137.7 \pm 881.3 a	698.7 \pm 212.5	105.5 \pm 23.5	56.3 \pm 7.8 a
	5	1326.7 \pm 297.7 a,b	543.0 \pm 158.9	131.0 \pm 37.9	72.0 \pm 33.1 a,b
	50	1578.5 \pm 352.3 b	618.0 \pm 264.9	170.2 \pm 47.6	110.4 \pm 48.7 b

These data represent the means \pm SD for groups of six mice. Values with different superscript letters in the same series differ significantly ($p < 0.05$).