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β-hydroxybutyrate suppresses pathological changes of blood-induced arthropathy in rats

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Arthropathy is a common complication in haemophilia and decreases quality of life. It has been known that concentrations of β-hydroxybutyrate (BHB) in blood are increased by a ketogenic diet, and elevated levels of circulating BHB restricts the progression of inflammation-mediated joint pathological changes. We hypothesized that elevation of blood BHB concentrations could be effective for reducing the progression of bleeding-induced arthropathy by moderating the inflammatory responses of macrophages. In this study, we investigated whether BHB alleviates the arthropathy caused by repeated intra-articular blood injection in rats. To increase blood BHB levels, rats were fed with ketogenic diet. Repeated intra-articular blood injection induced significant joint swelling, whereas ketogenic diet intake significantly increased blood BHB concentrations and ameliorated the joint swelling. The periarticular tissue-fibrosis observed in the control diet intake group appeared to be significantly alleviated in the ketogenic diet intake group. In addition, the IL-1β, which is involved in the progression of arthropathy, levels in the supernatants of blood-exposed macrophages derived from THP-1 cell line were significantly suppressed by BHB supplementation. In summary, BHB moderated the pathological joint changes caused by intra-articular blood exposure.

Arthritis and subsequent arthropathy are global health concern affecting a significant proportion of the population and associated with reduced quality of life. There are several causes of arthropathy, including rheumatoid arthritis (RA), osteoarthritis (OA), and haemarthrosis[1–3]. In each pathology, an inflammatory response is involved in the onset and progression of the disease. Recently, in vivo studies demonstrated that a ketogenic diet, comprising high-fat and low carbohydrate constituents, reduced the progression of inflammatory diseases by increasing the concentration of β-hydroxybutyrate (BHB) in circulating blood[4,5]. In addition, it has also been suggested that ketogenic diet and BHB may suppress the progression of arthritis associated with RA and OA[6–9]. In a preclinical study using a rat OA model, Ganggang et al. demonstrated that ketogenic diet ameliorated the progression of joint damage by suppressing inflammasome activity[5]. On the other hand, it is unclear whether the ketogenic diet would be effective for haemarthrosis caused by trauma or a bleeding disorder such as haemophilia.

Haemophilia is a bleeding disorder caused by a deficiency or defect of factor VIII (FVIII) or factor IX (FIX), and classified as haemophilia A and haemophilia B, respectively[10,11]. Patients with haemophilia (PwH) characteristically bleed into joints, not only after injury but also after physical exercise or even normal daily activities[12]. Joint bleeding is the most frequent manifestation of bleeding requiring treatment in both children and adults in severe PwH[12,13]. Joint bleeding leads to the synovial proliferation, in which fibroblast-like synoviocytes and macrophage-like synoviocytes produce proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. These cytokines further enhance the fibroblast-like synoviocyte proliferation. Repeated bleeding causes irreversible destruction of the joint, resulting in haemophilic arthropathy[14,15]. This clinical complication significantly reduces the patient's quality of life, with progression of the orthopaedic pathology frequently leading to joint replacement surgery[15]. Clinical studies have demonstrated that PwH treated with prophylactic therapy have less symptomatic bleeding and better joint conditions than those receiving on demand therapy with coagulation factor agents[16,17]. Nevertheless, some patients receiving prophylactic therapy continue to develop arthropathy that leads to impairment in mobility[16,17], and additional treatment can be

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needed to prevent progression of the dysfunctional lesion. In these circumstances, less invasive, oral treatment could offer substantial advantages.

There are several common aspects between haemophilic arthropathy and RA in the early stages of soft tissue changes and OA in the later stages of bone tissue changes[15]. As described above, the intra-articular level of IL-1β is elevated after intra-articular bleeding, and the inflammasome is involved in the release of IL-1β. Therefore, we considered that ketogenic diet might be beneficial to ameliorate bleeding-induced arthritis through an increase of blood BHB levels.

In this study, we investigated the relationship between circulating blood BHB levels and the extent of inflammation and subsequent joint damage using an autologous blood-induced arthropathy model in non-haemophilic wild type rats, where the amount of blood exposure in the joint can be precisely controlled. We used ketogenic diet intake to increase BHB concentration in the blood, which suppressed joint swelling and fibrosis of periarticular tissues caused by intra-articular blood exposure in in vivo studies. Elevation of blood BHB level is expected to be an effective treatment to inhibit the progression of haemophilic arthropathy.

Results

The suppressive effects of ketogenic diet on joint swelling induced by intra-articular blood injections were initially examined as described in Fig. 1a. The commencement of ketogenic diet feeding 1 week prior to the first intra blood injection (Day 0) resulted in significantly higher blood BHB concentrations compared to those in the control diet group, and these elevated circulating levels remained high throughout the experimental period (Fig. 1b). Repeated intra-articular blood injections caused significantly more joint swelling in the Blood + control diet group than in the Saline + control diet group, and these inflammatory responses were significantly suppressed in the Blood + ketogenic diet group (Fig. 1c). In addition, histological appearance and quantitation of the number of infiltrating mononuclear inflammatory cells in the fibrous-like tissue area surrounding the knee were evaluated at the end point (Day 26) (Fig. 2). A marked increase of fibrous-like tissue was evident in the Blood + control diet group (Fig. 2b,e) compared to the Saline + control diet group (Fig. 2a,d), and these distinct morphological changes, evident in the Blood + control diet group, were suppressed in the animals of Blood + ketogenic diet group (Fig. 2c,f). Moreover, the percentages of fibrous-like tissue seen in the infrapatellar fat pads measured using Image J, and the numbers of inflammatory mononuclear cells detected in the periarticular tissues were significantly increased in the Blood + control diet group compared to the Saline + control diet group, and these measurements were lower in the Blood + ketogenic diet group (Fig. 2j,k). There was no remarkable synovial iron deposition or cartilage destruction observed in any of the groups on Day 26 (Fig. 2g, h, and i). These data strongly indicated that the induced inflammatory responses might be alleviated by ketogenic diet. We investigated the mechanism by which the ketogenic diet suppressed the development of arthropathy by immunohistochemical analyses (Fig. 3). The number of inflammatory cytokine-positive cells (IL-1β, TNF-α, and IL-6) and inflammasome component protein-positive cells (ASC and NLRP3) surrounding knee joint were increased in the Blood + control diet group compared with in the Saline + control diet group. The number of these cells in the Blood + ketogenic diet group significantly decreased compared to the Blood + control diet group (Fig. 3p – t).

Studies were next extended to examine whether BHB was the primary mediator of the arthro-protective effects observed in ketogenic diet group. BHB was administered intraperitoneally 15 min before intra-articular blood injection and joint swelling was assessed 2 days after blood injection, as shown in Fig. 4a. The circulating BHB concentrations (Fig. 4b) significantly increased 15 min after administration (just before blood injection) (Group 2) compared to those administered with PBS (Group 1). Joint swelling was significantly reduced in the BHB-administered group compared to the PBS-administered group (Fig. 4c). In addition, we examined the effects of intra-articular injections of heterologous blood, to investigate the relative importance of circulating BHB or localized joint BHB for reducing joint swelling. In these experiments, the blood from BHB-administered rat (high BHB concentration blood) was injected into the right knee of the PBS-administered rat (Fig. 4a, Group 3), and alternatively, the blood from PBS-administered rat (normal BHB concentrations) was injected to the right knee of the BHB-administered rat (Fig. 4a, Group 4). As expected, circulating BHB levels in Group 4 were significantly higher than in Group 3 (Fig. 4b). The degree of joint swelling in both Group 3 and Group 4 was significantly reduced compared with Group 1 (Fig. 4c), indicating that elevated BHB concentrations either in the blood or in the joint, significantly moderated the inflammatory responses.

IL-1β is known to be involved in the development of haemophilic arthropathy, and macrophages provide a source for IL-1β release[14,15]. Furthermore, BHB has been shown to suppress IL-1β release from macrophages by inhibiting of the activity of the nucleotide-binding oligomerization, leucine-rich repeat, pyrin domain-containing protein 3 (NLRP3) inflammasome. Therefore, we investigated the effect of BHB on the IL-1β release induced by blood exposure. Macrophages derived from THP-1 by PMA treatment were cultured in medium in the presence of human blood. The concentrations of IL-1β in the culture supernatants were significantly enhanced 24 h after the addition of blood (Fig. 5a), and BHB significantly suppressed this increase (Fig. 5d). Similarly, levels of TNF-α and IL-6 in the culture supernatants were significantly increased after 24 h in the presence of human blood (Fig. 5b,c), but the addition of BHB had little or no obvious inhibitory effects on these measurements (Fig. 5e,f). The expression of IL-1β mRNA in these differentiated macrophages was also investigated and shown to be increased by the addition of blood, in a dose-dependent manner. BHB weakly suppressed this increase (Fig. 6a,b).

Discussion

The present findings demonstrated that BHB inhibited the pathological changes in knee joints induced by intra-articular injections of blood in our animal model. A rat model of blood-induced arthritis was created

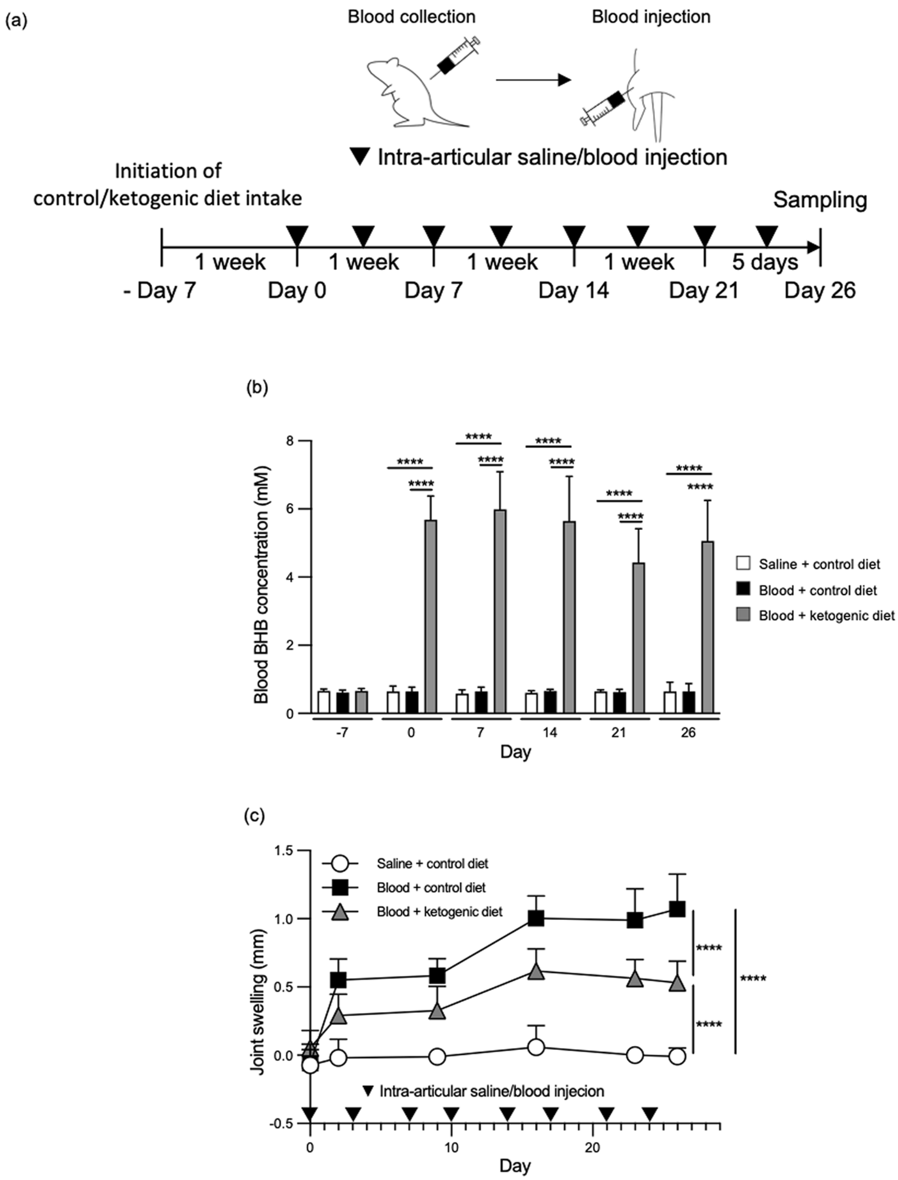


Fig. 1. Suppressive effects of ketogenic diet on joint swelling caused by repeated intra-articular blood injection. Control or ketogenic diet was commenced 7 days before the first intra-articular blood injection (Day 0). (**a**) Total of 8 intra-articular injections of autologous blood were performed twice a week from Day 0. Knee joints were assessed on Day 26. (**b**) β-hydroxybutyrate (BHB) concentrations in blood were measured on Days −7, 0, 7, 14, 21, and 26. (**c**) Joint swelling was evaluated before and after the initiation of intra-articular injection of autologous blood. The days of blood injection are shown as black triangles (**a** and **c**). In the bar graph and line plot (**b** and **c**), white identifies the intra-articular saline injection + control diet (Saline + control diet) group, black denotes the intra-articular blood injection + control diet (Blood + control diet) group, and gray illustrates the intra-articular blood injection + ketogenic diet (Blood + ketogenic diet) group. Data are shown as mean and SD ($n = 5–7$). Intergroup comparisons were assessed by two-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group (****$p < 0.0001$).

by injecting autologous blood into the joint twice-weekly for 4 weeks. In this rat model, a marked increase in fibrous tissue, inflammatory mononuclear cells, and degradation of proteoglycans were observed, but no cartilage destruction[18,19]. Ketogenic diet successfully reduced joint swelling and arthrofibrosis in this model (Figs. 1 and 2). Moreover, BHB administration reduced joint swelling even in the animals fed a standard chow (Fig. 4, Group 2), indicating that the arthroprotective effect of ketogenic diet was mainly provided by BHB.

To verify whether the arthroprotective effect of BHB was mediated by an increase in local intra-articular BHB concentrations, blood containing high concentrations of BHB was injected into the joints of rats with normal circulating levels of BHB, and the results demonstrated that joint swelling was reduced (Fig. 4). Less joint swelling was also observed when blood containing normal BHB concentrations was injected into the joints of rats with high levels of circulating BHB. Low molecular weight (MW) compounds, including BHB (104.1

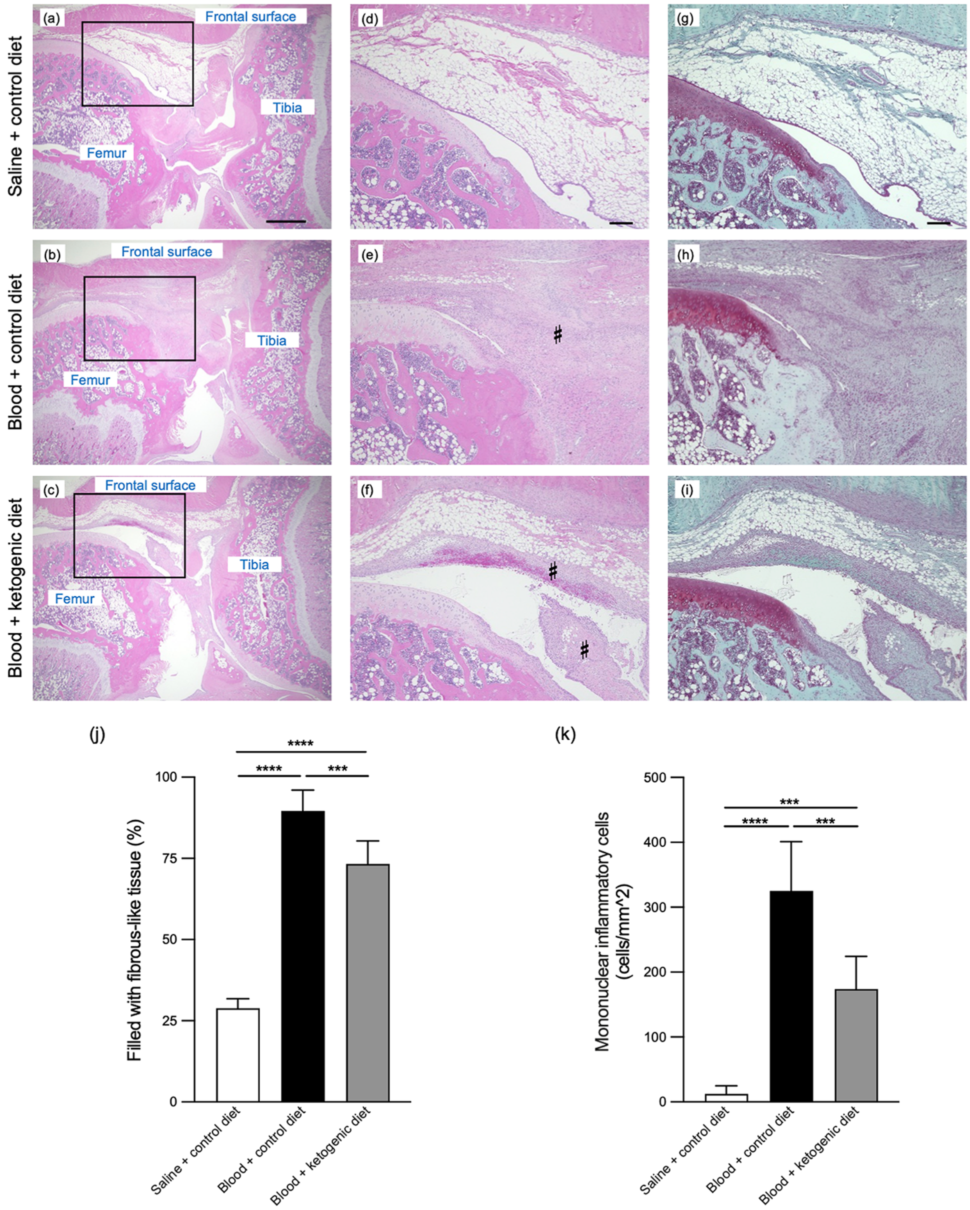


Fig. 2. Suppressive effects of ketogenic diet on arthrofibrosis and infiltration of mononuclear inflammatory cells after repeated intra-articular blood injections. The histology of knee joints on post intra-articular injection day 26 was compared, in the intra-articular saline injection + control diet (Saline + control diet) group (**a**, **d**, and **g**), the intra-articular blood injection + control diet (Blood + control diet) group (**b**, **e**, and **h**), and the intra-articular blood injection + ketogenic diet (Blood + ketogenic diet) group (**c**, **f**, and **i**) using haematoxylin-eosin and Safranin O-fast green staining. Representative low-power field images (**a**, **b**, and **c**), and selected high-power images of corresponding rectangular areas (**d**, **e**, **f**, **g**, **h**, and **i**) are illustrated. The scale bar in (**a**) indicates 1000 µm, and the scale bars in (**d**) and (**g**) indicate 200 µm. The frontal surface of the knee is located at the top in the images, the femur is on the left side, and tibia is at right side of the images. The percentage of fibrous-like tissue area (♯) in the infrapatellar fat pad (**j**) was measured for the evaluation of fibrosis using ImageJ software. The number of infiltrating inflammatory mononuclear cells per 1 mm^2 in the periarticular tissues was counted (**k**). White: Saline + control diet group, black: Blood + control diet group, and gray: Blood + ketogenic diet group (**j** and **k**). Data are shown as mean and SD. Intergroup comparisons were assessed by one-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group ($^{***}p < 0.001$, $^{****}p < 0.0001$).

Da), undergo unimpeded transport by passive diffusion in the synovium[20]. The arthroprotective effects in these rats seemed likely to be due, therefore, to increased BHB concentrations in the joints as a result of the transfer of BHB from the circulating blood, and our data supported the hypothesis that increased intra-articular BHB concentrations are pivotal for joint protection.

After bleeding, some factors such as heme and damage-associated molecular patterns (DAMPs) in red blood cells activate inflammasome leading to release of IL-1β[21,22]. It has been reported that inflammatory cytokines, including IL-1β, were elevated in the joint after the induction of intra-articular bleeding in haemophilic mice[23]. Previous in vivo and in vitro studies have demonstrated that BHB inhibited NLRP3 inflammasome activity and resulted in reduced IL-1β release[4,5,24–26]. Our current studies focused, therefore, on the relationship between IL-1β and the arthroprotective effect of BHB. Indeed, our in vivo study showed that a ketogenic diet decreased the number of IL-1β cytokine-positive cells and inflammasome component protein-positive cells surrounding the knee joint (Fig. 3). In addition, our in vitro study showed that BHB directly reduced the release of IL-1β from blood-exposed macrophages differentiated from THP-1 cells while not reducing IL-1β mRNA expression. Our findings along with previous reports suggest that BHB might suppress the increase in IL-1β positive cells and inflammasome component positive cells, and suppress IL-1β release from intra-articular macrophages by inhibiting inflammasome activity in joint (Figs. 3p, q and r, 5d and 6b). In our study, we focused on whether the ketogenic diet and BHB inhibit the progression of arthropathy induced by blood exposure, and further studies are needed to elucidate the detailed mechanisms mainly inflammasome activity in the future.

Although there are other derivatives of ketone bodies, such as acetoacetate and butyrate, only BHB, but not the other derivatives, has a function inhibiting the LPS/ATP-induced inflammasome activity[24]. In addition, there are two enantiomers in BHB (R-BHB and non-oxidizable chiral enantiomer S-BHB); however, we used R-BHB to evaluate because R-BHB is the normal product of human metabolism[27]. On the other hand, Goldberg et al. S-BHB inhibited IL-1β secretion from neutrophils stimulated by LPS/ATP[4]. Therefore, S-BHB might exert an arthroprotective effect in our model.

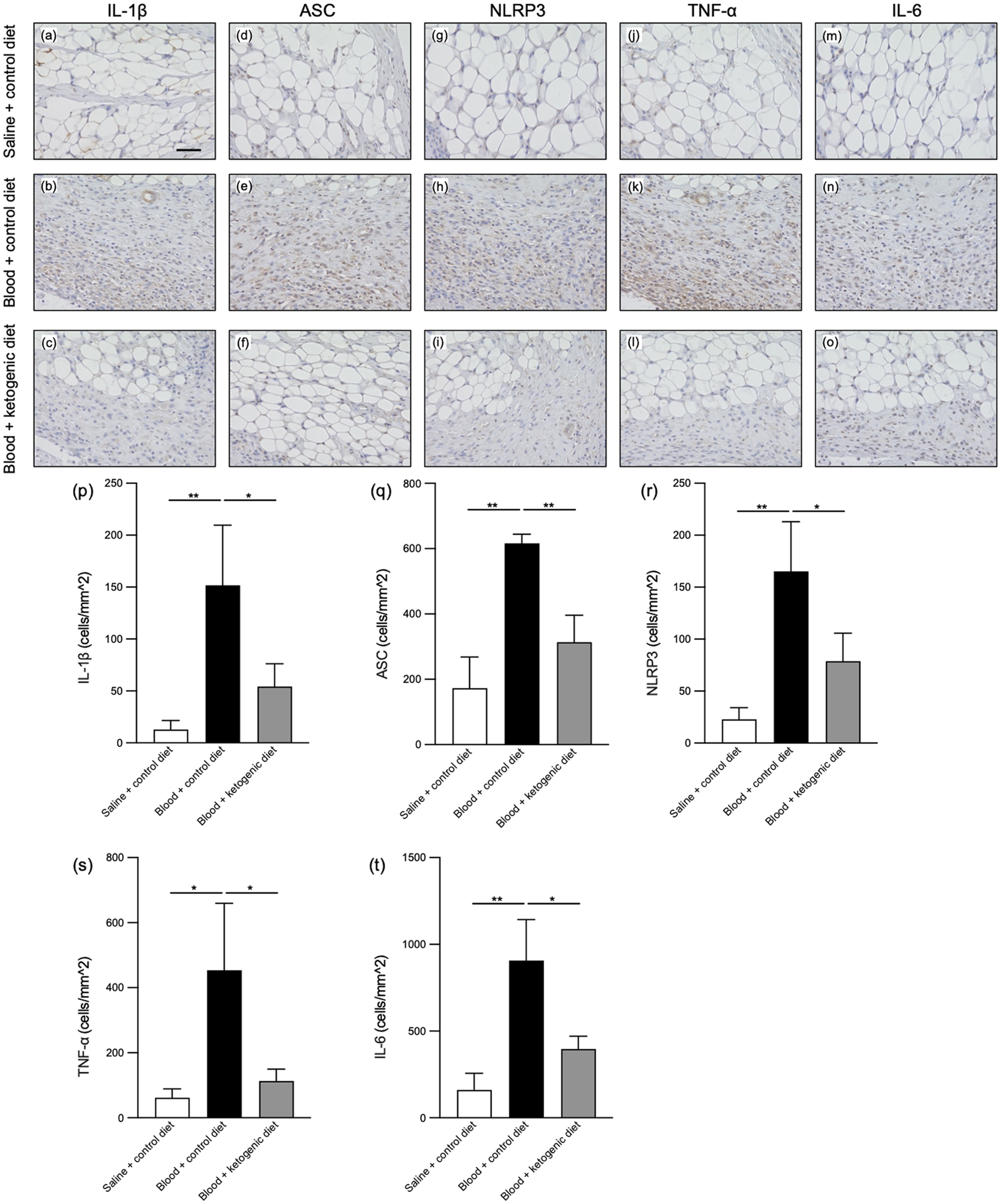
TNF-α and IL-6 are also known to be inflammatory cytokines involved in the development of haemophilic arthropathy[14,15], and previous reports demonstrated that inhibition of TNF-α or IL-6 alleviated blood-induced arthropathy in FVIII knock out mice in vivo[28,29]. In our in vivo study, the decrease in the number of elevated TNF-α and IL-6 positive cells caused by ketogenic diet intake might lead to the arthroprotective effect (Fig. 3s,t). In this context, therefore, the present investigations were extended to evaluate the effects of blood and BHB supplementation on TNF-α and IL-6. As the result, BHB did not appear to have an inhibitory effect on TNF-α and IL-6 release in THP-1 derived macrophages. Indeed, IL-1β increases the expression of TNF-α and IL-6[30]. In our in vitro study, the influence of blood exposure to increase in TNF-α and IL-6 might be greater than the inhibitory effect of BHB on IL-1β release.

Haemophilia is an inherited disease and therefore requires treatment from paediatric age. Ketogenic diet therapy appears promising for the prevention of haemophilic arthropathy; however, long-term intake may have difficult consequences, especially in paediatric patients. For example, while ketogenic diet has been shown to be effective in drug-resistant epilepsy, adverse side effects including vomiting and diarrhoea have been identified, and there are concerns about growth in children because of imbalanced nutritional ketogenic diet[31,32]. Intermittent, on-demand ketogenic diet therapy could be an advisable alternative, therefore, where the ketogenic diet is commenced a few days before and continued temporarily after physical activity in which bleeding is likely to occur. Alternatively, some studies have investigated the safety and kinetics of oral intake of ketone mono ester ((R)-3-hydroxybutyl (R)-3-hydroxybutyrate) and diester (bis-hexanoyl-(R)-1,3-butanediol), and have demonstrated an increase in BHB concentrations in circulating blood[33,34]. Our results confirmed that BHB reduced joint swelling in animals maintained on a standard chow, and it may be that an arthroprotective effect could be mediated by these different compounds. Blood BHB concentrations in our experiments were higher than those in the alternative clinical trials, however, and the optimum concentrations needed for joint protection whilst avoiding side effects of ketogenic diet require further confirmation.

This present study applied an animal model to examine potential orthopaedic consequences in response to localized bleeding, and the limitations with this type of investigation are well recognized. In particular, in the present context, the blood-induced arthropathy experiments were non-haemophilic, and the findings did not reflect the effect of BHB on joint pathology in the absence of FVIII or FIX. We recognize the need to conduct future experiments using FVIII or FIX deficient animal. In addition, the impact of ketogenic diet and BHB on haemosiderin deposition and its associated responses were difficult to determine in these short-term experiments. Haemosiderin is believed to induce IL-1β release and chondrocyte death, leading to cartilage damage[35,36]. Finally, our attempts to collect synovial fluid for measuring BHB and cytokines concentrations in joint fluid were unsuccessful, and the transfer of BHB from circulating blood to joint fluid remained speculative. Nevertheless, our current findings suggested that the maintenance of high BHB concentrations in circulating blood might be an effective option to prevent the arthropathy caused by intra-articular bleeding in haemophilia patients.

Conclusion
We have shown that an increase in blood BHB concentrations, mediated by ketogenic diet intake or BHB administration, suppresses the inflammatory response of blood-induced arthritis. These results provide evidence that could lead to the development of new treatments to protect against joint destruction in current haemophilia patients who rarely experience symptomatic bleeding. If oral intake can enhance joint prognosis, they are anticipated to improve the quality of life for patients with bleeding-induced arthropathy, such as those with haemophilia, while minimizing additional burden.

Methods
Animals
All animal experiments were approved by the Animal Care Committee of Nara Medical University in accordance with the policies established in the NIH Guide for the Care and Use of Laboratory Animals (Permit No. 13010



and 13109) and in compliance with the ARRIVE guidelines. Male Wistar rats (6 and 7 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were housed in polycarbonate cages with bedding, free access to water and experimental diets under a constant 12-hour light/12-hour dark cycle. All animals were fed a conventional laboratory chow diet until the initiation of experiments.

Induction of arthritis

The arthritis rat model was designed using a modified protocol described in a previous report[18]. Intra-articular injections of autologous blood were administered 8 times, twice a week from the age of 8 weeks. Animals anesthetized prior to injection using 3% isoflurane (Pfizer Inc., New York, USA). Blood was obtained from the

◀**Fig. 3**. Suppressive effects of ketogenic diet on the number of inflammatory cytokine-positive cells and inflammasome component protein-positive cells surrounding knee joint after repeated intra-articular blood injections. The immunohistochemical staining of knee joints on post intra-articular injection day 26 was compared, in the intra-articular saline injection + control diet (Saline + control diet) group (**a**, **d**, **g**, **j**, and **m**), the intra-articular blood injection + control diet (Blood + control diet) group (**b**, **e**, **h**, **k**, and **n**), and the intra-articular blood injection + ketogenic diet (Blood + ketogenic diet) group (**c**, **f**, **i**, **l**, and **o**) using anti-IL-1β antibody (**a** – **c**), anti-ASC antibody (**d** – **f**), anti-NLRP3 antibody (**g** – **i**), anti-TNF-α antibody (**j** – **l**), and anti-IL-6 antibody (**m** – **o**). The scale bar in (**a**) indicates 50 μm. The number of inflammatory cytokine-positive cells (IL-1β, TNF-α, and IL-6) and inflammasome component protein-positive cells (ASC and NLRP3) per 1 mm^2 in the periarticular tissues was counted (**p** – **t**). White: Saline + control diet group, black: Blood + control diet group, and gray: Blood + ketogenic diet group (**p** – **t**). Data are shown as mean and SD. Intergroup comparisons were assessed by one-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group (*$p < 0.05$, **$p < 0.01$).

subclavian vein without the use of anti-coagulant using a 26 G needle (volume: 0.3 ml), and 0.1 mL was then immediately injected into the right knee joint of the same animal (autologous blood injection) using a 30 G needle. Control rats were injected with same volume of saline. The control diet [carbohydrate: 80%, protein: 10%, fat: 10%]; (D10070802, Research Diets, Inc., New Brunswick, USA) or ketogenic diet [carbohydrate: 0%, protein: 10%, fat: 90%]; (D10070801, Research Diets, Inc.) was commenced seven days before Day 0. These arthropathy experiments were designed to include 3 groups of animals ($n = 5–7$, each group): saline injection + control diet feeding (Saline + control diet), blood injection + control diet feeding (Blood + control diet) and blood injection + ketogenic diet feeding (Blood + ketogenic diet) groups. Levels of circulating BHB were measured in blood drawn from tail veins using an electrode (FreeStyle, Abbott Japan LLC, Minato-ku, Japan). As an indicator of acute inflammatory response, joint swelling was assessed by measuring medio-lateral diameters using a calliper under isoflurane anaesthesia two days after each intra-articular injection of blood or saline. The degree of joint swelling was calculated by comparing the width of the left knee joint (non-injected) with that of the right knee joint (injected).

Histological assessment

Knee joint histology was assessed on day 26 after saline or blood injection into joint cavity. Rats were sacrificed by exsanguination under anaesthesia with isoflurane. The knee joints were removed, skinned and post-fixed in formalin, prior to decalcification (EDTA solution, for 1 month), paraffin-embedding, sagittal section cutting (3–5 μm). Fibrosis around the knee joint was evaluated after staining with haematoxylin-eosin and Safranin O-fast green as previously described[37,38]. The area infiltrated by fibrous-like tissue in the infrapatellar fat pad was quantified using ImageJ software (National Institutes of Health, MD, USA). The number of mononuclear inflammatory cells per 1 mm^2 evident in the periarticular tissue was counted manually. Assessments were directed by a skilled pathologist in Nara Medical University.

Immunohistochemistry assessment

For immunohistochemical staining, the specimens were incubated with primary antibodies against IL-1β (1:100, 26048-1-AP, Proteintech, Rosemont, USA), IL-6 (1:100, GTX110527, GeneTex, Irvine, USA), TNF-α (1:200, NBP1-19532, Novus, Centennial, USA), NLRP3 (1:100, SAB5700723, Sigma-Aldrich, Saint Louis, USA), and ASC (1:100, bs-6741R, Bioss, Woburn, USA) at 4 °C overnight. After washing with TBS, the slides were incubated with goat anti-rabbit HRP-conjugated secondary antibody (SignalStain Boost IHC Detection Reagent, Cell Signaling, Danvers, USA). Finally, the reactions were developed using 3'3-diaminobenzidine. The number of the positively stained cells per 1 mm^2 was counted manually. We assessed $n = 3$ in each group (Saline + control diet group, Blood + control diet group, and Blood + ketogenic diet group).

The assessment of BHB effects on the progression of arthropathy

Rats (8 weeks old, fed with a chow diet) were administered with a single intraperitoneal (i.p.) dose of phosphate-buffered saline (PBS) or BHB (8 mmol/kg) (Toronto Research Chemicals INC., Toronto, Canada) under isoflurane anaesthesia. Intra-articular blood was then infused as described above after 15 min. Blood BHB concentrations were measured immediately before the intra-articular injection. A total of 4 groups were used in this experiment ($n = 5–7$, each group) (Fig. 4a). Groups 1 included rats initially administered PBS, and subsequently injected with autologous blood into the knee joint. Group 2 included rats initially administered BHB, and subsequently injected with autologous blood into the knee joint. Group 3 included initially PBS-administered rats, and subsequently injected with blood from BHB-administered rats (Group 4: high concentrations of circulating BHB) into the knee joint. Conversely, group 4 included initially BHB-administered rats, and subsequently injected with the blood from PBS-administered rats (Group 3: blood with normal BHB concentrations) into the knee joint. Joint swelling was assessed two days after the intra-articular injections.

Cell culture

The monocytic leukaemia cell line, THP-1, was purchased from JCRB Cell Bank (Osaka, Japan), and the cells were incubated with RPMI1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% foetal bovine serum (FBS) (MP-Biomedicals, Irvine, USA), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Waltham, USA), and 30 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Saint Louis, USA) for 96 h[39] to induce macrophage formation. The differentiated macrophages were then incubated in

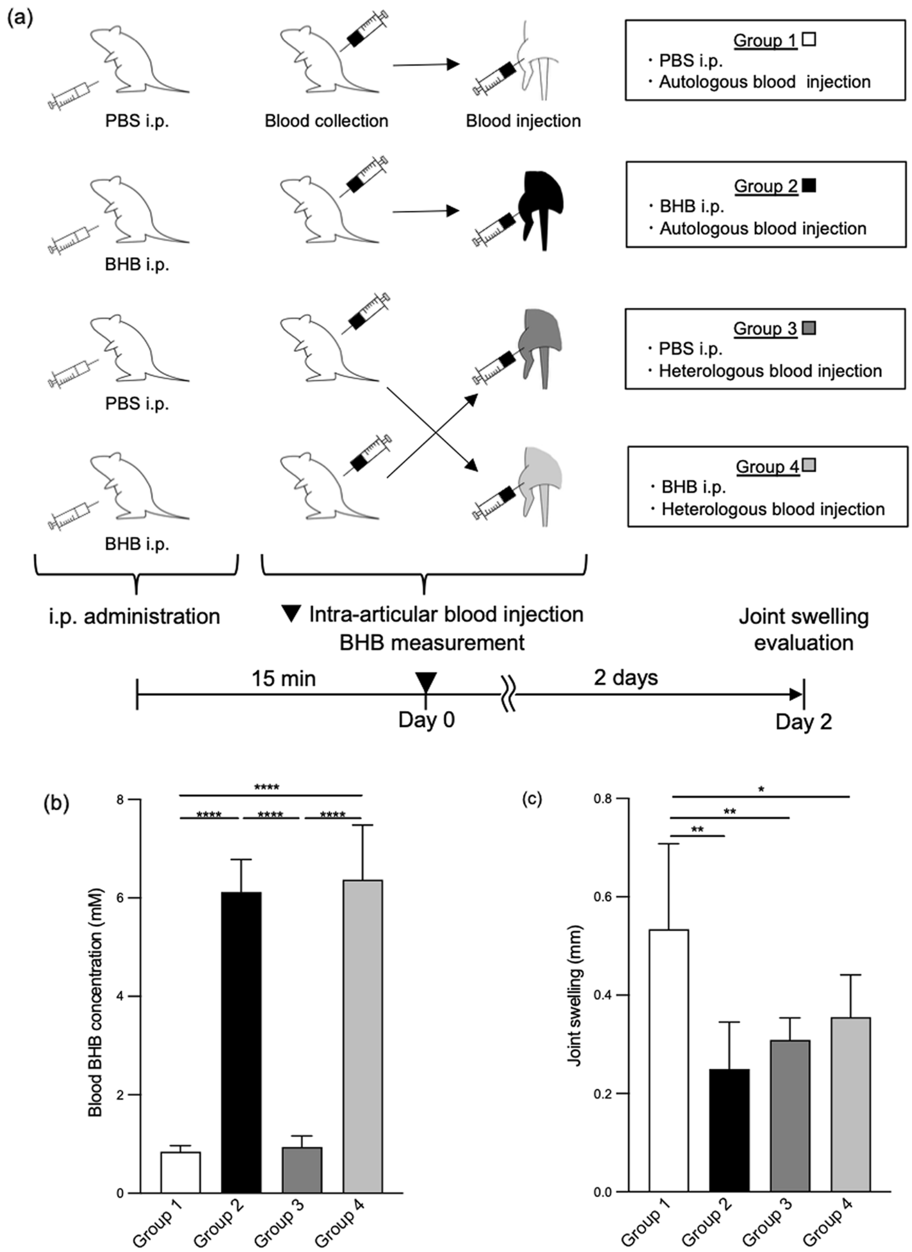


Fig. 4. Direct effects of BHB on suppressing joint swelling caused by intra-articular blood injection. Rats fed with a chow diet were administered single intraperitoneal (i.p.) injection of PBS or BHB (8 mmol/kg) under isoflurane anaesthesia followed fifteen minutes by an intra-articular injection of whole blood. (**a**) Different groups of animals were assigned ($n = 5$–7 in each group) to receive autologous blood (Group 1 and 2), or blood with normal concentrations of BHB and with high concentrations of BHB (Group 3 and 4) as described in methods. (**b**) BHB concentrations were measured before the intra-articular blood injections. (**c**) Joint swelling was assessed two days after the blood injections. White: Group 1, black: Group 2, gray: Group 3, and light gray: Group 4 (**a**, **b**, and **c**). Data are shown as mean and SD. Intergroup comparisons were assessed by one-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

medium with or without 1 ~ 10% human blood purchased from BioIVT (Westbury, USA) in the presence of BHB (final concentration 1 ~ 10 mM) or PBS. Culture supernatants and cells were collected after 24 h and stored at − 145 °C for the assessment of cytokine excretion as described below.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of human IL-1β, TNF-α, and IL-6 in cell culture supernatants were measured using Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, USA) according to the manufacturer's instructions.

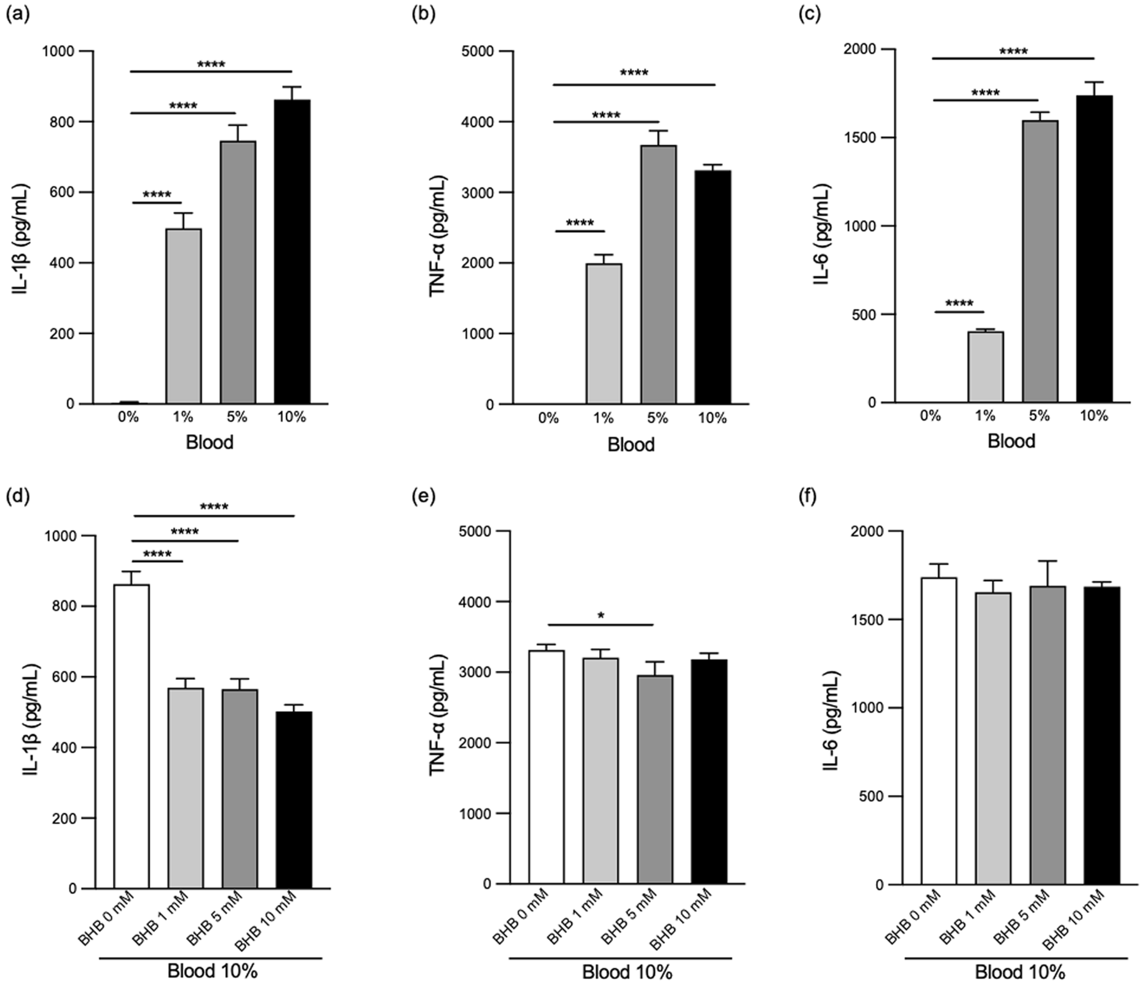


Fig. 5. Direct effects of BHB on suppressing IL-1β excretion from macrophages differentiated from the THP-1 cell line. THP-1 cells were incubated in RPMI1640 medium containing 30 nM Phorbol 12-myristate 13-acetate (PMA) for 96 h to initiate macrophage differentiation. After induction, cells incubated in RPMI1640 medium with or without 1 ~ 10% human blood for 24 h prior to quantitation of IL-1β, TNF-α, and IL-6 by ELISA (**a**, **b**, and **c**). BHB (1 ~ 10 mM) or PBS was added to wells containing 10% blood (**d**, **e**, and **f**). Data are shown as mean and SD. Intergroup comparisons were assessed by one-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group (*$p < 0.05$, ****$p < 0.0001$).

Quantitative polymerase chain reaction (qPCR)

Total cellular RNA was extracted using NucleoSpin RNA Plus (Machery-Nagel GmbH & Co. KG, Duren, Germany), and 0.4 µg was reverse-transcribed to cDNA using a High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, USA). The mRNA expression levels of various genes were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) together with StepOne Plus (Thermo Fisher Scientific) techniques. The relative expression levels of IL-1β were calculated relative to peptidylprolyl isomerase A (PPIA, the normalizer) using the comparative cycle threshold method. Gene primers and probes were purchased from Thermo Fisher Scientific (TaqMan Gene Expression Assay. IL-1β: Hs01555410_m1, PPIA: Hs04194521_s1).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.3.1 software (GraphPad Software, San Diego, USA). Intergroup comparisons were assessed by one-way ANOVA or two-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group. All values are shown as mean and standard division (SD). P value of < 0.05 was considered statistically significant.

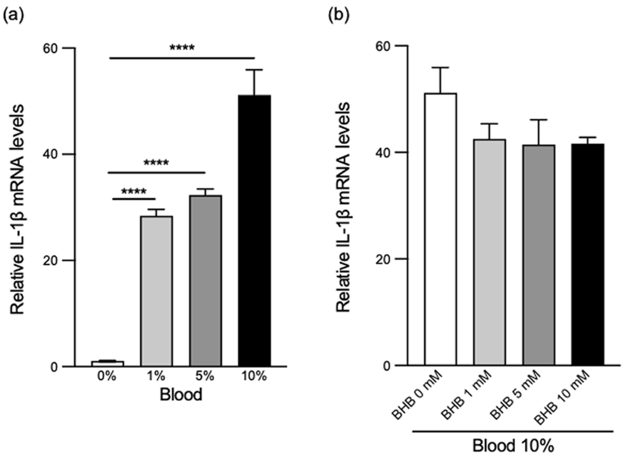


Fig. 6. Effects of BHB on the expression of IL-1β in macrophages differentiated from THP-1 cells. (**a**) THP-1-derived macrophages were incubated in RPMI1640 medium with or without 1 ~ 10% blood, and IL-1β mRNA expression was quantified by qPCR 24 h after blood addition. (**b**) BHB was added to wells containing 10% blood. Data are shown as mean and SD. Intergroup comparisons were assessed by one-way ANOVA. Bonferroni multiple comparison test was used for comparisons among each group (****$p < 0.0001$).

Data availability
The datasets generated during and/or analysed during the current study are available in the figshare repository, https://figshare.com/articles/dataset/BHB_ketogenic_diet_arthropathy_rat/27247050?file=49844778.

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Declarations

Competing interests
RK, AS, KT, NM, SH, TS, YN, and MS: members of Medical Biology of Thrombosis and Hemostasis established by Nara Medical University and Chugai Pharmaceutical Co., Ltd.RK, NM, SH, TS, YN, and YY: employees of Chugai Pharmaceutical Co., Ltd.RK, NM, SH, and YY: stock ownership of Chugai Pharmaceutical Co., Ltd.TS, YN, YY, and MS: Patents for inventions relating to products of Chugai Pharmaceutical Co., Ltd.AS: speaker's bureau from CSL Behring.KT: grants or research support from Japan Blood Products Organization, The Mother and Child Health Foundation and Novo Nordisk Pharma.SM, MT, and SK: no competing interest. MS: representative of Medical Biology of Thrombosis and Hemostasis collaborative research laboratory.; research support from Chugai Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd. and CSL Behring.; honoraria or consultation fees from Chugai Pharmaceutical Co., Ltd.; speaker's bureau from Chugai Pharmaceutical Co., Ltd., CSL Behring, Sanofi, Bayer, Novo Nordisk Pharma, Takeda Pharmaceutical Co., Ltd., Pfizer and Fujimoto Seiyaku Corp. RK, NM, SH, TS, YN, and YY: employees of Chugai Pharmaceutical Co., Ltd. RK, NM, SH, and YY: stock ownership of Chugai Pharmaceutical Co., Ltd. TS, YN, YY, and MS: Patents for inventions relating to products of Chugai Pharmaceutical Co., Ltd. AS: speaker's bureau from CSL Behring. KT: grants or research support from Japan Blood Products Organization, The Mother and Child Health Foundation and Novo Nordisk Pharma. SM, MT, and SK: no competing interest. MS: representative of Medical

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