DOI 10.1002/mnfr.200500027 Mol. Nutr. Food Res. 2005, *49*, 772–778

Dietary supplement of isohumulones inhibits the formation of aberrant crypt foci with a concomitant decrease in prostaglandin E2 level in rat colon

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Male Fischer 344 rats were subcutaneously injected with azoxymethane (AOM) twice weekly at a dose of 15 mg/kg and were fed with freeze-dried (FD) samples of beer brewed without hops (non-hops beer), beer with hops at 4 times the amount of regular lager beer (× 4-hops beer), and isomerized hop extract (IHE) for the whole experimental period (I/PI) or for the post-initiation period (PI) only. Feeding FD beer samples at a dose of 1% significantly decreased the number of aberrant cryp foci (ACF) in the PI protocol over five weeks. × 4-hops beer showed stronger inhibitory effects on the development of the numbers of aberrant crypts per focus and large ACF with four or more crypts than non-hops beer. Feeding IHE to rats at a dose of 0.01% or 0.05% in either the I/PI or PI experiment significantly reduced the numbers of ACF. Prostaglandin E2 (PGE2) levels in colonic mucosa of AOM-treated rats were significantly reduced by feeding of IHE. PGE2 production induced by lipopolysaccharide/interferon-γ (LPS/IFN-γ) in RAW264.7 cells was also reduced by treatment with IHE and isohumulone in a dose-dependent manner. These observations suggest that isohumulones show chemopreventive effects on ACF formation in rat colon by inhibiting the production of PGE2.

Keywords: Aberrant crypt foci / Chemoprevention / Hops / Isohumulones / Prostaglandin E2

Received: February 28, 2005; revised: April 27, 2005; accepted: April 28, 2005

1 Introduction

It has been demonstrated by epidemiological and experimental studies that dietary habit may affect the incidence of colon cancer, one of the most prevalent neoplastic diseases. The western lifestyle with diets high in fat and low in vegetable consumption are definitive determinants which may increase the incidence of colon cancer. It has been reported that many dietary factors may prevent the incidence of colon cancer. Such dietary factors, including polyphenols [1, 2], dietary fiber [3], polyunsaturated fatty acids [4, 5], and lactic acid bacteria [6], show chemopreventive effects.

Our previous study has demonstrated that beer intake inhibits carcinogenesis in rat colon induced by azoxymethane (AOM), and that the effects are due to ingredients of beer derived from its raw materials, namely barley malts and hops [7]. With regard to the association between beer consumption and colorectal cancer risk in an epidemiological study, some revealed inverse relations [8], but the others reported positive relations [9–11]. Beer is the only alcoholic beverage that contains hops, the female inflorescences of the hop plant (*Humulus lupulus* L.), and they are used as an essential raw material for beer brewing to enhance bitterness, aroma, and antimicrobial activity. Many physiological functions related to chemoprevention have been reported for the chemical components of hops. For example, the induction of phase II enzymes by prenylated chalcones [12, 13], inhibition of cyclooxygenase-2 (COX-2) by humulone [14], inhibition of angiogenesis by humulone [15], induction of apoptosis by humulone [16, 17], anti-inflammatory effects by prenylated chalcones [18, 19], and agonistic effects on peroxisome proliferator-activated receptors

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Abbreviations: **ACF**, aberrant crypt foci; **AIN**, AIN-76A; **AOM**, azoxymethane; **COX-2**, cyclooxygenase-2; **EIA**, enzyme immunoassay; **FD**, freeze-dried; **IH**, isohumulone; **IHE**, isomerized hops extract; **I/PI**, initiation and post-initiation; **LPS/IFN-γ**, lipopolysaccharide/interferon-γ; **MTT**, 3-[4,5-dimethylthyazol-2-yl]-2,5-diphenyltetrazoliumbromide; **PGE2**, prostaglandin E2; **PPAR**, peroxisome proliferator-activated receptor

(PPARs) α and γ by isohumulones [20]. Therefore, it is plausible that ingredients in beer derived from hops are responsible for the chemopreventive effects of beer.

The most abundant ingredients in beer derived from hops are isohumulones, which are the major bitter components and found in beer at concentrations of 10 – 100 ppm. Isohumulones comprise three structurally related compounds, isohumulone, isocohumulone, and isoadhumulone. They are converted from humulones present in hops by isomerization during the wort boiling step of the brewing process. Isomerized hop extract (IHE), which primarily contains these three compounds, has been approved for human consumption and is used for beer production. Yajima *et al.* [20] recently demonstrated that isohumulones act as dual agonists of PPARs α and γ in transient activation assays *in vitro*. Furthermore, ingestion of isohumulones ameliorates insulin resistance in diabetic mice, and supplementation of the diet of diabetic patients with IHE for 8 weeks reduce their plasma glucose and hemoglobin A1c levels [20]. It is also reported that isohumulones increase plasma high-density lipoprotein (HDL)-cholesterol levels with concomitant reduction in the atherosclerosis index in mice fed an atherogenic (high-fat and high-cholesterol) diet [21]. These observations suggest that intake of isohumulones may have some health benefits by modulating the genes under the control of PPARs α and γ *in vivo*.

Aberrant crypt foci (ACF) are the early hyperproliferative lesions and are putative preneoplastic lesions for colon cancer [22 – 25]. It is the convenient and established method to evaluate the ACF for the prediction of the chemopreventive efficacy in the short-term animal experiments. In the present study, we investigated the effects of ingredients from hops in beer and isohumulones on AOM-induced rat ACF formation and prostaglandin E2 (PGE2) production *in vivo* and *in vitro*. Our study demonstrates that isohumulones exert a chemopreventive effect; they suppressed the formation of ACF and PGE2 production in colonic mucosa and PGE2 production from lipopolysaccharide (LPS)-induced macrophage cells.

2 Materials and methods

2.1 Materials

AOM and LPS were purchased from Sigma Chemical (St. Louis, MO, USA). IFN-γ, 10% neutral-buffered formalin, and methylene blue were purchased from Wako Pure Chemical (Osaka, Japan) and piroxicam from ICN Biomedicals (Costa Mesa, CA, USA). RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). IHE, containing 30% w/w isohumulones, was purchased from English Hop Products (Kent, UK). The purity of isohumulones in IHE is 79% and the ratio of isohumulone : isocohumulone : isoadhumulone in IHE is 37 : 48 : 15. Isohumulone was purified from IHE as reported previously [20]. Two beers brewed in pilot scale were used in the present experiment, namely beer brewed without hops (non-hops beer) and beer brewed with up to a 4-fold larger amount of hops (× 4-hops beer).

2.2 Experimental samples

Freeze-dried (FD) beer sample was mixed with the AIN-76A basal diet (Dyets Inc., Bethlehem, PA, USA) at 1%. Cornstarch was added to the control diets to mimic isocaloric conditions, since most of the calories in the solid component of beer are derived from carbohydrates. IHE was mixed in the AIN-76A diet at 0.01%, 0.04%, and 0.05% in the experiment. Piroxicam was used as a control to compare the efficacy of the inhibitory effect and was added to the basal diet at a concentration of 200 ppm, as used in a previous report [7, 26].

2.3 Experimental procedures for ACF assays

The animals were treated in accordance with Kirin Pharmaceutical's ethical guidelines for animal care, handling, and termination. Male Fischer 344 rats, 4 weeks old, were purchased from Charles River Japan and maintained in wire cages in an air-conditioned room under constant conditions of temperature (22 ± 2°C) and humidity (55 ± 10%). The animals were divided into experimental groups after 4 days of acclimatization. ACF formation was investigated by two protocols, as indicated in Fig. 1. Test samples were given to the animals during the whole experimental period (initiation/post initiation I/PI), or during the post-initiation phase (PI). For the PI protocol, experimental diets were given three days after the second AOM injection until the end of the experiment (Figs. 1b, c). In all protocols, 1 week after the start of experiments, rats were treated with subcutaneous injections of AOM (15 mg/kg body weight) once a week for 2 weeks. FD beer was mixed in the basal diet, AIN76-A, at 1%. IHE was also mixed in AIN76-A at 0.01% and 0.05%. Ingested amounts of the experimental diets were recorded to estimate the consumption of the test materials. All rats were weighed weekly until the end of the experimental period, at which point the animals were sacrificed and colons were excised for subsequent analysis of ACF. After fixation in 10% buffered formalin, colons were stained with 0.2% methylene blue and ACF were scored under a light microscope at × 40 magnification. ACF were distinguished from normal crypts by their increased size, more prominent epithelial cells, and their increased pericryptal area [25]. The number of ACF and the number of aberrant crypts (ACs) per rat were quantified.

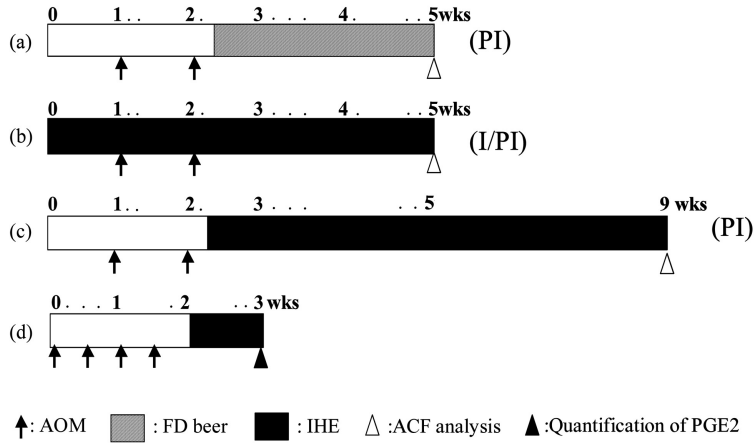

Figure 1. Experimental protocols for the treatment of animals used. Five-week old male F344 rats were used and sacrificed at the end of the experimental periods. Animals received subcutaneous injections of AOM at the indicated time points shown by arrows. In the ACF experiments, rats received experimental diets for either the whole 5-week period (I/PI protocol) or from three days after the second AOM injection until the end of the experiment (PI protocol). In the evaluation of PGE2 production in colonic mucosa, feeding of experimental diets started three days after the final AOM injection and continued for one week.

2.4 PGE2 production in murine macrophage RAW264.7

RAW 264.7 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cell concentrations were adjusted to 5×10^5 cells/mL and 200 μL was seeded in each well of a 96-well plate. After 1 h of incubation, cells were treated with LPS (100 ng/mL), IFN-γ (100 units/mL), and test samples dissolved in DMSO (final DMSO concentration 0.2%, v/v). In the present experiment, isohumulone was examined at concentrations of 5 and 2.5 μg/mL, and IHE was examined at concentration of 25 and 10 μg/mL. After 16 h of treatment, the levels of PGE2 in the supernatant were measured using the Prostaglandin E2 EIA System (Amersham Biosciences, Tokyo, Japan) according to the manufacturer's directions. The PGE2 production by vehicle treatment (0 μg/mL treatment of IH or IHE) was caluculated as 100% production and the relative rate on PGE2 production by sample treatment was calculated by comparison with vehicle treatment. Cytotoxicity was measured by 3-[4,5-dimethylthyazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay.

2.5 PGE2 production in colonic mucosa

PGE2 production in colonic mucosa of rats was examined using the protocol reported previously [27]. Male Fischer 344 rats, 5 weeks old, were treated with subcutaneous injections of AOM (15 mg/kg body weight) twice a week for 2 weeks as indicated in Fig. 1d. AIN-76A diets containing 0.01% and 0.04% IHE were given to rats three days after the final AOM injection. One week after feeding of the experimental diets, all rats were sacrificed. Colons were excised and washed with PBS, then they were scraped by a glass slide to remove mucosa. Scraped mucosa were suspended in PBS and homogenized. After centrifugation, the supernatant was examined for PGE2 content using the protocol described above.

2.6 Statistical analysis

Data are expressed as means ± SD. The data in the *in vivo* experiment were analyzed by 1-way analysis of variance (ANOVA) proceeded by Dunnett's test or Kruskal-Wallis test and finally followed by the Bonferroni Correction. Significant differences were concluded when a P value of less than 0.05 was obtained.

3 Results

3.1 General observations

The body weights of rats and ingestion of diets in the control and experimental groups were not significantly different. Chronic administration of FD beer or IHE was not associated with any distinct changes or signs of toxicity.

3.2 Inhibitory effects of FD beer on AOM-induced ACF formation

As shown in Table 1, a significant decrease in the number of ACF was observed by feeding of FD non-hops and × 4-hops beer samples (30 and 31% reduction, respectively). The suppressive effects of FD beer samples on ACF formation were almost of the same magnitude as those observed when feeding with FD beer with a regular amount of hops [7]. Although the inhibitory effect of FD × 4-hops beer on the number of ACF was almost the same as that of FD non-hops beer, stronger inhibitory effects on the average ACF size and the number of large ACF (ACF with four or more crypts/focus) were observed in rats fed on FD × 4-hops beer.

3.3 Inhibitory effects of isohumulones on PGE2 production by murine macrophage RAW264.7

IHE and isohumulone dose-dependently inhibited the production of PGE2 induced by LPS/IFN-γ in murine macro-

Table 1. Effects of FD beer on AOM-induced ACF formation (PI, 5W)

Group	Incidence	Body weight (g)	Daily diet intake (g/rat)	No. of ACF/colon	No. of aberrant crypts/colon	No. of aberrant crypts/focus	No. of ACF 4AC/colon
AOM + AIN	8/8	199.6	10.9	115.3 ± 22.2[a)]	240.1 ± 48.6	2.09 ± 0.19	7.6 ± 4.2
AOM + 1% FD hops(−) beer	8/8	199.9	10.8	80.5 ± 17.4[c)]	160.6 ± 37.2[d)]	1.99 ± 0.15	3.8 ± 2.9[b)]
AOM + 1% FD hops(×4) beer	8/8	198.0	10.9	79.3 ± 16.3[d)]	149.8 ± 32.1[d)]	1.89 ± 0.12[b)]	2.3 ± 2.0[c)]

a) Mean ± SD
b)–d) Significantly different from AOM + AIN group: b) $p < 0.05$; c) $p < 0.01$; d) $p < 0.005$

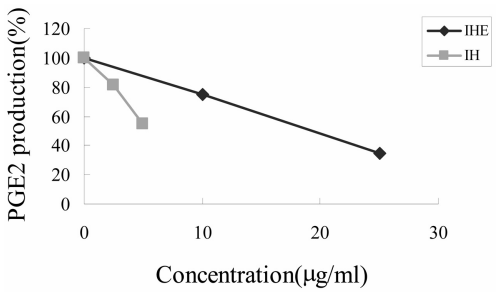

Figure 2. Effects of IHE and IH on PGE2 production from murine macrophage RAW264.7 cells induced by LPS/IFN-γ. Cells were treated with LPS (100 ng/mL), IFN-γ (100 units/mL), and test samples dissolved in DMSO (final DMSO concentration, 0.2%, v/v) for 16 h. The PGE2 production by vehicle treatment (0 μg/ml treatment of IH or IHE) was caluculated as 100% production and the relative rate on PGE2 production by sample treatment was calculated by comparison with vehicle treatment.

phage RAW264.7 as shown in Fig. 2. The MTT assays indicated that cell viabilities for IH treatment are 94.8% and 96.3% when the cells were treated at concentrations of 2.5 and 5 μg/mL, respectively. IHE treatment also did not affect the cell viability (107.0% and 105.5% for 10 and 25 μg/mL, respectively.) IH inhibited the production of PGE2 around 20% and 50% when the cells were treated with concentrations of 2.5 and 5 μg/mL, respectively. IHE at concentrations of 10 and 25 μg/mL inhibited the production of PGE2 around 25% and 65%, respectively (equivalent to 3.3 and 7.5 μg isohumulones/mL).

3.4 Inhibitory effects of IHE on AOM-induced ACF formation (I/PI, 5W)

Intake of IHE during the whole experimental period of 5 weeks (I/PI) significantly reduced the number of ACF/colon by 28% and 40% at doses of 0.01% and 0.05%, respectively (Table 2). The number of ACs/colon was also significantly reduced by intake of IHE (31% and 43% reduction for doses of 0.01% and 0.05%, respectively). The number of large ACF was significantly reduced by intake of 0.05% IHE (63% reduction). Piroxicam, a cyclooxygenase inhibitor, significantly reduced the number of ACF, ACs, and large ACF in the same manner as observed previously [7, 26].

3.5 Inhibitory effects of IHE on AOM-induced ACF formation (PI, 9W)

Intake of IHE at a dose of 0.01% in the basal diet during the PI period (9W) significantly reduced the number of ACF/colon by 35% (Table 3). The reduction produced by the intake of a dose of 0.05% IHE was not statistically significant. The number of large ACF was dose-dependently reduced by intake of IHE, but the reduction was not statistically significant. Average ACF size was not significantly changed in any of the groups including the piroxicam group.

3.6 Inhibitory effects of PGE2 production by IHE in colonic mucosa induced by AOM

Treatment of AOM at a dose of 15 mg/kg, 4 times in two weeks, elevated the PGE2 level in colonic mucosa to approximately two times that of the saline-treated group. Feeding of IHE at doses of 0.01% and 0.04% for 1 week significantly reduced the PGE2 level in colonic mucosa. Ingestion of piroxicam also significantly reduced the PGE2 level, to the basal level.

4 Discussion

Our previous study has demonstrated that beer intake inhibits carcinogenesis in rat colon induced by AOM, and that the effects are due to ingredients of beer derived from its raw materials, barley malts and hops [7]. Although a significant inhibitory effect on AOM-induced colonic carcinogenesis was demonstrated for malts in the study, it was not ascertained whether hops are also responsible for the chemoprevention. The comparison in this study of the effect on ACF formation of two types of beer, non-hops beer and ×4-hops beer, indicates that beer ingredients from hops

Table 2. Effect of IHE on AOM-induced ACF formation (I/PI, 5W)

Group	Incidence	Body weight (g)	Daily diet intake (g/rat)	No. of ACF/colon	No. of aberrant crypts/colon	No. of aberrant crypts/focus	No. of ACF 4AC/colon
AOM + AIN	8/8	211.1	12.1	77.3 ± 21.5[a)]	175.5 ± 48.9	2.27 ± 0.13	8.1 ± 5.0
AOM + 0.01% IHE	8/8	201.7	11.7	55.3 ± 17.2[b)]	120.5 ± 37.6[b)]	2.19 ± 0.13	4.0 ± 2.0
AOM + 0.05% IHE	8/8	204.5	11.9	46.4 ± 12.4[d)]	99.1 ± 25.7[d)]	2.15 ± 0.14	3.0 ± 1.7[b)]
AOM + 0.02% piroxicam	8/8	204.7	11.4	27.3 ± 9.0[e)]	58.3 ± 20.5[e)]	2.13 ± 0.26	1.9 ± 1.5[e)]

a) Mean ± SD
b)–d) Significantly different from AOM + AIN group: b) $p < 0.05$; c) $p < 0.01$; d) $p < 0.005$; (e) $p < 0.001$

Table 3. Effects of hops extract on AOM-induced ACF formation (PI, 9W)

Group	Incidence	Body weight (g)	Daily diet intake (g/rat)	No. of ACF/colon	No. of aberrant crypts/colon	No. of aberrant crypts/focus	No. of ACF 4AC/colon
AOM + AIN	8/8	267.4	12.7	70.3 ± 13.2[a)]	203.3 ± 54.3	2.89 ± 0.29	19.1 ± 9.8
AOM + 0.01% IHE	8/8	273.9	13.3	45.6 ± 11.1[b)]	143.9 ± 44.1	3.16 ± 0.44	16.4 ± 10.1
AOM + 0.05% IHE	8/8	273.4	12.8	56.1 ± 20.2	160.6 ± 55.6	2.86 ± 0.22	12.7 ± 4.9
AOM + 0.02% piroxicam	8/8	275.7	13.2	26.7 ± 6.7[d)]	73.3 ± 21.6[d)]	2.74 ± 0.30	5.1 ± 3.6[c)]

a) Mean ± SD
b)–d) Significantly different from AOM + AIN group: b) $p < 0.05$; c) $p < 0.005$; d) $p < 0.001$

might be responsible for the inhibition of both growth of initiated cells and foci development in colonic mucosa (Table 1). Furthermore, FD × 4-hops beer showed stronger inhibitory effects on the average ACF size and the number of large ACF (ACF with four or more crypts/focus) than non-hops beer, again suggesting that ingredients from hops might inhibit the growth of initiated cells and retard foci development in colonic mucosa.

Feeding of IHE, mainly consisting of isohumulones, during the whole experimental period (I/PI) or after the carcinogen treatment (PI), exerted a significant inhibition on ACF formation (Tables 2, 3). LPS/IFN-γ-induced PGE2 production in RAW264.7 was inhibited by the treatment with IHE or IH, indicating that IH suppresses the inflammatory responses caused by LPS/IFN-γ treatment (Fig.2). Furthermore, PGE2 levels in colonic mucosa from the AOM-treated rats were significantly reduced by feeding rats the diets containing IHE for one week (Fig. 3). This is the first observation to indicate that isohumulones inhibit the production of PGE2. There are two isoforms of COXs, COX-1 and COX-2, related to the production of PGE2. COX-2 is hardly expressed under normal physiological conditions, but it is dramatically induced by stimulation with cytokines, growth factors, and bacterial endotoxins, physiological states associated with chronic inflammatory disease or carcinogenesis [28]. Many dietary factors exert inhibitory effects of COX-2, including flavonoids [29, 30], sesquiterpenoids [31], herbal medicines [32], *etc*. It was reported that humulone from hops potently inhibited the transcription of the COX-2 gene and catalytic activity of COX-2 enzyme, and thus prevented angiogenesis [14, 15]. These observations suggest that isohumulones in beer, which are converted

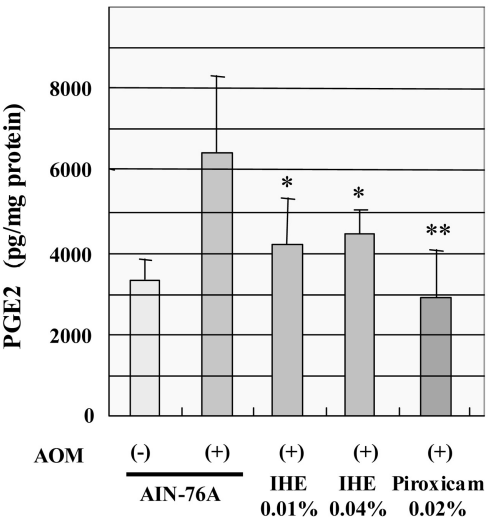

Figure 3. Effects of IHE on PGE2 production in colonic mucosa induced by AOM. Experimental diets were fed to the animals (n = 4) for 1 week after the final injection of AOM. Colons were excised and the amount of PGE2 was measured by enzyme immunoassay (EIA). Bars indicate means ± SD values. Statistical significance comparison results between experimental diet groups and control group (AIN76A diet treated with AOM) are shown by * for $P < 0.05$ and ** for $P < 0.01$, respectively.

from humulones during beer production, also exert their chemopreventive effect by inhibiting COX-2 production.

Recent evidence demonstrates that there is a close relationship between the activation of PPAR γ and inhibition of the expression of COX-2 in cancer chemoprevention. PPAR γ agonists are likely to downregulate the expression of

COX-2 [33–36] and prevent chemically induced carcinogenesis in mammary and colon [37–40]. It was also demonstrated that COX-2 inhibitors and PPAR γ ligands synergistically modulate tumor cell growth to inhibit cancer development more effectively than targeting each molecule alone [38, 41]. Isohumulones were recently identified as dual agonists for PPAR α and γ and improve insulin-independent diabetes and disorders of lipid metabolism [20, 21]. It is possible that the agonistic effect of isohumulones on PPAR-γ results in the downregulation of COX-2, which in turn reduces PGE2 levels and thus reduces the number of neoplastic legions (ACF).

In conclusion, our data show that beer components derived from hops inhibit the development of ACF in rat colon, and that isohumulones are partly responsible for the chemopreventive properties. Isohumulones inhibit PGE2 production induced by LPS/IFNγ in RAW264.7 cells and reduce PGE2 levels in colonic mucosa in AOM-treated rats. Further experiments are required to clarify the underlying mechanism of chemopreventive activities of isohumulones.

We would like to thank Dr. Odai for technical support in the experiments. We are also grateful to K. Akatsuka for animal care and the brewing department of Kirin Brewery Co., Ltd. for supplying the special beers brewed in pilot scale.

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