

# Inhibitory effects of Cheonggukjang extracts on radiation-induced micronucleus formation and inflammasome activation

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## ABSTRACT

### ► Original article

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**Background:** People are exposed to more radiation than before with the application of radiation technology. Radiation is known to induce damage to cell structure, DNA, chromosomes and nucleus. In this study, we showed that CGJ extract can inhibit radiation-induced chromosomal damage in vivo and NLRP7 inflammasome activation in vitro, suggesting that the compound from CGJ can be considered as a therapeutic materials to reduce adverse effect inflammation and chromosome aberration during radio-therapy. **Materials and methods:** In this study the inhibitory effects of Cheonggukjang (CGJ) extract on the radiation-induced micronucleus formation in vivo and the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activation in vitro. **Results:** We observed the prolonged presence of radiation-induced inflammation and its onsecutive damage in hematopoietic tissues after irradiation by examining micronucleus formation in polychromatic erythrocytes. Mouse bone marrow-derived macrophages were used to investigate NLRP3 inflammasome activation. The micronucleus analysis with mouse peripheral polychromatic erythrocytes using acridine orange staining showed a significantly ( $p < 0.05$ ) reduced frequency of micronucleated bone marrow polychromatic erythrocytes (MnPCEs) in CGJ-treated mice. **Conclusion:** CGJ extract elevated the chance that mice would survive a potentially lethal dose of gamma-irradiation. Additionally, The CGJ extract attenuated IL-1 $\beta$  maturation by interrupting the inflammasome. The CGJ extract attenuates radiation-induced micronucleus formation possibly mediated by inhibiting inflammasome, which provides valuable information for the further study of the mechanism of action of CGJ extract.

**Keywords:** Cheonggukjang, radiation, micronuclei, inflammasome, NLRP3.

## INTRODUCTION

The application of radiation technology is increasingly extensive with the development of

science and technology. Therefore humans are being exposed to radiation more than before [1]. Radiation is known to induce damage to cell structure, DNA, chromosomes and nucleus.

Radiation-induced reactive oxygen species (ROS) and the inflammation resulting from their creation are increasingly recognized as important factors in radiation damage, especially at the chromosomal level <sup>(2)</sup>. Inflammatory mediators act together in perpetuating and amplifying the inflammation cascade. They suppress DNA repair mechanisms, leading to microsatellite instability and chromosomal instability, culminating in abnormal chromosomal segregation (micronucleus formation) and aneuploidy <sup>(2)</sup>. Gamma-radiation can increase ROS levels, resulting in IL-1 $\beta$  expression. Functionally, ROS were proposed to be exclusively involved in inflammasome activation <sup>(3,4)</sup>.

The inflammasome is a multiprotein complex in myeloid cells that serves as a platform for caspase-1 activation, resulting in interleukin-1 $\beta$  (IL-1 $\beta$ ) maturation. IL-1 $\beta$ , a pro-inflammatory cytokine, is the most important of all cytokines because of its central role in the inflammatory process. Once the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is activated, it converts inactive pro-IL-1 $\beta$  into the bioactive and secreted forms of IL-1 $\beta$  <sup>(5,6)</sup>.

Cheonggukjang (CGJ) is traditionally made by fermenting whole cooked soybeans with *Bacillus subtilis* spp. for short periods (6 days) without the addition of salt. CGJ was reported various physiological effects including antioxidant, anti-inflammatory, anti-hypertensive, anti-obesity, anti-diabetic, and cholesterol-lowering activities <sup>(7-12)</sup>. Recently, our collaborators reported that poly-gamma-glutamic acid from CGJ compound can regulate the inflammasome activation <sup>(13)</sup>. However, it is still unknown whether CGJ can inhibit inflammasome activation to suppress the radiation-induced micronucleus formation.

In this study, we showed that CGJ extract can inhibit radiation-induced chromosomal damage *in vivo* and NLRP3 inflammasome activation *in vitro*, suggesting that the compound from CGJ can be considered as a therapeutic materials to reduce adverse effect inflammation and chromosome aberration during radio-therapy.

## MATERIALS AND METHODS

### Animals

All experiments were carried out with 4 to 8 week-old male BALB/C mice weighing 14-20g (Orient Bio, Gyeonggi-Do, Korea). The animals were bred and maintained at 25 $\pm$ 2°C on a standard mouse diet (Lab Diet, St. Louis, MO, USA). All animal experiments were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-130325-1).

### Preparation of CGJ extract

CGJ and its extract were prepared at the Korea Food Research Institute by the traditional processing method. Briefly, white beans were submerged in water at 15°C for 15h before being steamed in a commercial steamer (NK, Eunkwang Machinery, Korea) at a pressure of 1.7kg/cm<sup>2</sup> for 30min. The beans were then cooled to 50°C and subsequently fermented with rice straw at 42°C for 40h in a fermentation room. Samples of CGJ produced by the fermenting beans were obtained 0, 5, 10, 20, and 40h after the start of the fermentation process. The concentration of the final CGJ extract was adjusted to 100mg/ml with distilled water. The CGJ samples were then freeze-dried and stored at 80°C.

### Treatment of mice with CGJ extract

A total of 200mg CGJ extract/kg body weight was administered by gavage (2ml/kg diluted with 8 ml/kg normal saline per animal) to the experimental animals for 20 days. The animals were irradiated 1 week after the final feeding.

### Irradiation

Whole-body irradiation of the experimental animals was carried out in a gamma chamber (Gammacell 40 Exactor, Ottawa, Ontario, Canada) obtained from the Radiation Health Research Institute, Korea Hydro and Nuclear Power Co., Ltd. The animals were exposed to 2Gy gamma radiation for micronucleus analysis and to 7Gy gamma radiation for survival-rate measurement.

### Body and spleen weights

In this study, the body weight is expressed as the rate of weight gain after irradiation. The spleen weight is expressed as a proportion of the body weight (spleen mass [g]/body mass [g]).

### Measurement of leukocytes, erythrocytes, and thrombocytes

Whole blood was collected from the subclavian artery and vein while the mice were under ether anesthesia. The hematologic analysis was performed by using VetScan HM5 hematology analyzer (Abaxis, USA) to measure the hematologic parameters containing the numbers of leukocytes, erythrocytes, and thrombocytes.

### Micronucleus analysis

We analyzed micronuclei to assess chromosomal damage. Briefly, acridine orange (Merck, Germany) was dissolved in distilled water (1mg/ml), and 10 $\mu$ l of the solution was placed on pre-heated (~70°C) clean glass microscope slides. Approximately 3 $\mu$ l of peripheral blood was obtained from the subclavian vein of each mouse. Micronuclei in polychromatic erythrocytes (MnPCEs) were counted using a fluorescence microscope (NIKON E-600, B-2A), and at least 1,000 polychromatic erythrocytes in the peripheral blood were scored per mouse for each data point.

MnPCEs with more than 75% of peak hemoglobin levels were counted 24h after irradiation. Counting MnPCEs in that way ensured that the micronuclei were induced directly by the irradiation rather than by aging or other factors. Five mice were included in each experimental group (control and CGJ extract-treated groups with no irradiation or with 2Gy radiation).

### Differentiation of bone marrow-derived macrophages (BMDMs)

Primary BMDMs were cultured and differentiated *in vitro* as described previously [13]. Briefly, BMDMs were derived from femoral and tibia bone-marrow progenitors. The progenitors were cultured in Dulbecco's modified Eagle medium (PAA Laboratories, GE

Healthcare Bio-Science Co., NJ, USA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories), 100  $\mu$ g/ml penicillin/streptomycin (PAA Laboratories), and 30% L929 cell-conditioned medium as a source of granulocyte/macrophage colony-stimulating factor. The cells were seeded in non-tissue culture-treated petri dishes and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 7 days.

### Inflammasome activation

BMDMs (1.0 $\times$ 10<sup>6</sup> cells per well) were plated on 12-well plates and primed with 10 $\mu$ g/ml lipopolysaccharide (LPS; Sigma-Aldrich Co., MO, USA) in RPMI 1640 medium containing 10% FBS and antibiotics for 3h. After LPS priming, NLRP3 was activated in the BMDMs by one of several reagents: ATP (ATP; 2mM, 1h, InvivoGen, CA, USA), nigericin (nigericin; 40M, 1h, Tocris Bioscience, Bristol, UK), aluminum potassium sulfate (Alum; 2mg/ml, 3h; Daejung Chemicals & Metals Co., Gyeonggi-do, Korea), or CaCl<sub>2</sub> (CaCl<sub>2</sub>; 1mM, 1h, Biosesang, Seoul, Korea). The BMDMs were exposed to the reagents for 1h or 3h (as indicated) to activate the NLRP3 inflammasomes, and supernatants, lysates, and pellet samples were subsequently collected for further analysis.

### Western blot analysis

The supernatants, lysates, and pellet samples were separated by SDS-PAGE on 10% or 16% polyacrylamide gels. After electrophoresis, the separated proteins were transferred to PVDF membranes. The membranes were probed overnight at 4°C with anti-mouse IL-1 $\beta$  antibody (R&D Systems, MN, USA) or anti-actin antibody (Santa Cruz Biotechnology). The membranes were further probed with HRP-conjugated-secondary antibodies and visualized using a Power-Opti ECL solution (Bionote Co., Gyeonggi-do, Korea).

### Statistical analysis

All data are presented as the mean  $\pm$  SD. We compared data between the two groups using the nonparametric Mann-Whitney U test with a significance level of  $p < 0.05$ .

## RESULTS

### Effects of CGJ extract on Body and spleen weights and blood cells

There was no significant difference in the rate of body weight gain and spleen to body weight between the mice after irradiation with and without CGJ extract (table 1).

The CGJ extract had no significant effect on the red blood cells, white blood cells, or platelets (table 2).

### CGJ extract inhibits micronucleus formation

Micronuclei arise from acentric fragments or centric chromosomes left behind during mitosis because of chromosome breakage or defects in the spindle apparatus caused by radiation-induced inflammation. To investigate the inhibitory effect of CGJ extract on micronucleus formation, peripheral blood was stained with acridine orange after exposure to 2Gy gamma-radiation. An analysis of micronuclei in polychromatic erythrocytes was then performed using a fluorescence microscope (figure 1A). The number of micronuclei per 1,000 polychromatic erythrocytes was measured and compared with that in control cells (figure 1B).

The data indicate that the CGJ extract had a protective effect against radiation-induced chromosomal damage, suggesting that CGJ extract is an anticlastogenic agent.

### CGJ extract elevates the survival rate following irradiation

The survival rate among the mice was measured after total-body irradiation with or without prior administration of CGJ extracts (figure 2). The mice were exposed to 7 Gy alone or in combination with CGJ extract as described above. Survival was defined as the time from irradiation to the time of death. Mice that

received CGJ extract before irradiation had an increased chance of surviving a potentially lethal dose of radiation. Only a few mice survived a radiation dose of 7Gy without receiving CGJ extract, and half of the mice that did not receive CGJ extract died within 12 days of irradiation. The CGJ extract provided a significant protective effect against the irradiation.

CGJ extract given before irradiation elevated the chance that the mice would survive a potentially lethal dose 7Gy of gamma-radiation.

### CGJ extract inhibits NLRP3 inflammasome activation

To investigate the effect of CGJ extract on inflammasome activation, we first administered various dosages of the extract (2.5–10%) to the LPS-primed BMDMs with or without ATP, a well-known activator of the NLRP3 inflammasome. As shown in figure 3A, the CGJ extract alone did not inhibit IL-1 $\beta$  (p17) maturation or alter pro-IL-1 $\beta$  secretion into the cell supernatant in the absence of ATP. When it was co-administered with ATP, however, the extract attenuated IL-1 $\beta$  (p17) secretion in a dose-dependent manner. Active IL-1 $\beta$  (p17) was not present in the cell lysate, indicating that the CGJ extract blocked IL-1 $\beta$  maturation but not secretion.

We further confirmed the inhibitory effects of the extract on the other NLRP3 inflammasome activators, such as nigericin, CaCl<sub>2</sub> and Alum. We activated NLRP3 inflammasomes with the three activators in the LPS-primed BMDMs in the presence of various concentrations of CGJ extract. As shown in Figure 3B, the extract significantly attenuated IL-1 $\beta$  (p17) secretion in a dose-dependent manner in the macrophages treated with nigericin, CaCl<sub>2</sub> or Alum, whereas the inhibitory potency of the CGJ extract on NLRP3 inflammasome activation varied.

**Table 1.** B.W. gain and spleen percentage values in mice after irradiation with and without CGJ extract.

	Cheonggukjang (2Gy)	Control (2Gy)
Rate of B.W.gain	1.083 $\pm$ 0.02 <sup>NS</sup>	0.977 $\pm$ 0.03
Spleen (g% B.W)	0.199 $\pm$ 0.02 <sup>NS</sup>	0.204 $\pm$ 0.02

Values are mean $\pm$ SD.

NS: not significant versus control group by Mann-Whitney U test.



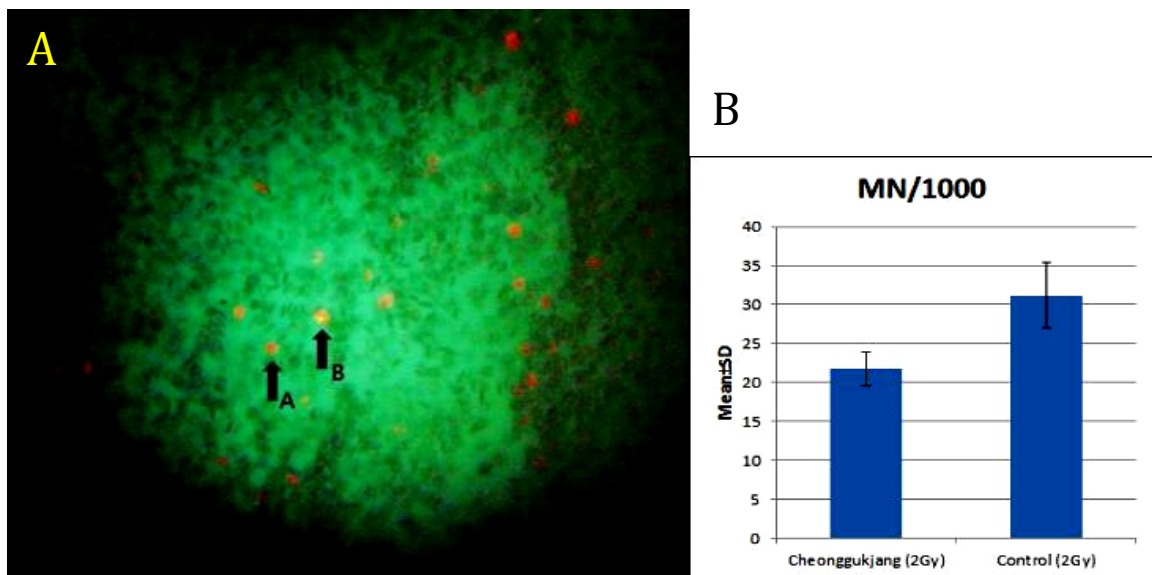
**Table 2.** Hematological values of mice after irradiation with and without CGJ extract

		Cheonggukjang (2Gy)	Control (2Gy)
<b>Leukocytes</b>	WBC( $10^9/L$ )	$1.39 \pm 0.97^{NS)}$	$0.65 \pm 0.38$
	LYM( $10^9/L$ )	$0.90 \pm 0.70^{NS)}$	$0.39 \pm 0.22$
	MON( $10^9/L$ )	$0.12 \pm 0.11^{NS)}$	$0.05 \pm 0.04$
	NEU( $10^{10}/L$ )	$0.37 \pm 0.17^{NS)}$	$0.21 \pm 0.16$
	RBC( $10^{12}/L$ )	$7.45 \pm 2.62^{NS)}$	$9.08 \pm 0.33$
	HGB(g/dl)	$11.46 \pm 4.48^{NS)}$	$14.88 \pm 0.59$
	HCT%	$34.66 \pm 11.93^{NS)}$	$44.91 \pm 1.34$
<b>Erythrocytes</b>	MCV(fl)	$46.60 \pm 0.55^{NS)}$	$49.60 \pm 0.55$
	MCH(pg)	$15.16 \pm 1.24^{NS)}$	$16.40 \pm 0.45$
	MCHC(g/dl)	$32.54 \pm 2.71^{NS)}$	$33.14 \pm 0.68$
	RDW%	$18.08 \pm 0.55^{NS)}$	$19.96 \pm 0.52$
	PLT( $10^9/L$ )	$220.60 \pm 131.13^{NS)}$	$354.50 \pm 87.91$
<b>Thrombocyte</b>	MPV(fl)	$8.76 \pm 1.17^{NS)}$	$8.12 \pm 0.52$
	PDW%	$34.84 \pm 3.38^{NS)}$	$32.08 \pm 3.91$

Values are mean $\pm$ SD.

NS: not significant versus control group by Mann-Whitney U test.

WBC: white blood cell, LYM: lymphocyte, MON: monocyte, NEU: neutrophil, RBC: red blood cell, HGB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, PLT: platelet, MPV: mean platelet volume, PDW: platelet distribution width.



**Figure 1.** Polychromatic erythrocytes stained with acridine orange (AO) and micronucleus analysis **A)** A-normal polychromatic erythrocyte. B-micronucleated polychromatic erythrocyte. **B)** The administration of CGJ extract significantly reduced the number of micronuclei induced by 2Gy gamma-radiation. (Administered group: mean $\pm$ SD = 21.74 $\pm$ 2.15, Control group: mean = 31.20 $\pm$ 4.21,  $p < 0.05$ )

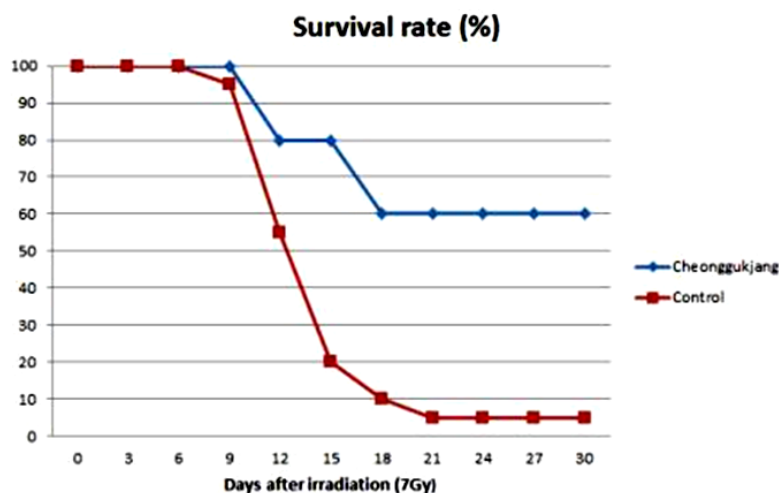
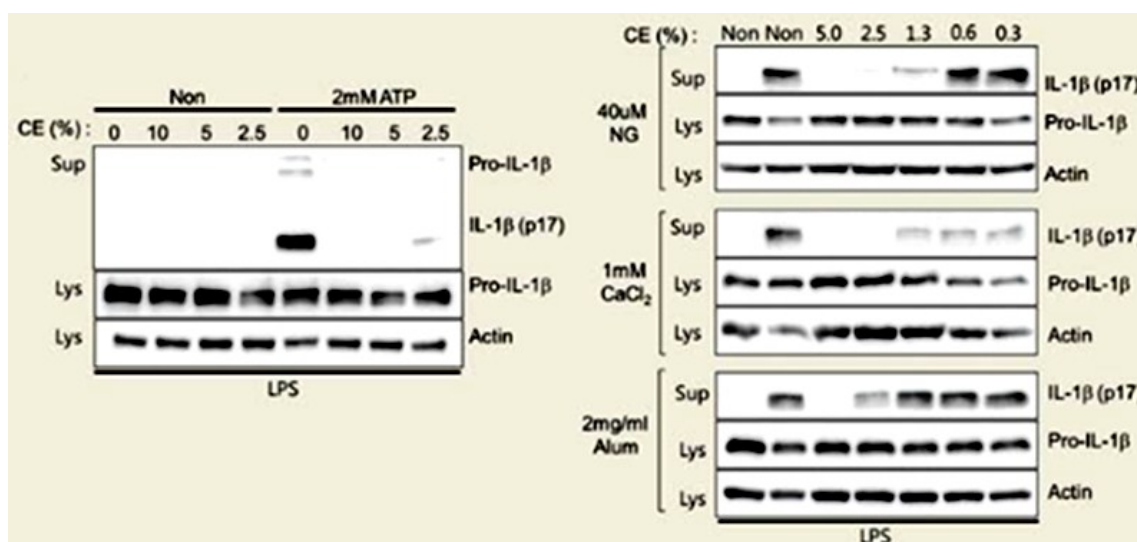


Figure 2. Survival rates of the mice after irradiation with and without CGJ extract



**Figure 3.** Effects of CGJ extract on NLRP3 inflammasome activation BMDMs (1cell/well) were primed with LPS (10g/ml) in RPMI medium containing 10% FBS and antibiotics for 3h. **A)** The cells were placed in RPMI medium with the indicated percentage of CGJ extract (CE) in the absence or presence of ATP (2mM), and the cellular supernatant (Sup) and lysate (Lys) collected after 1h.

**B)** LPS-primed BMDMs were treated with nigericin (40mM) and CaCl<sub>2</sub> (1mM) for 1h, or with Alum (200mg/ml) for 3h in the presence of the indicated concentration of CE. The cellular supernatants and lysates were analyzed with the indicated antisera by an immunoblot assay.

## DISCUSSION

It is well known that high dose of ionizing radiation is sufficient to disrupt DNA, leading to an increased risk of gene toxicity. However, we have not yet found a clear risk about low-dose ionizing radiation. Our results indicate that low dose of irradiation has no effect on body weight, biochemical markers and blood. Similar data have been reported previously [14]. Radiation-induced inflammation was reflected

in the frequency of polychromatic erythrocytes, with higher numbers of micronuclei and chromosomal aberrations in the irradiated cells compared with those in control cells. There was a significant decrease in the percentage of micronucleated polychromatic erythrocytes after gamma irradiation in mice that were pretreated with CGJ extract compared with that in mice that were not pretreated with CGJ extract. CGJ extract given before irradiation increased the chance that the mice would

survive a potentially lethal dose of total-body irradiation. Without the CGJ extract, only a few mice survived a radiation high-dose of 7 Gy, and half of the irradiated mice died within 12 days after of irradiation. The CGJ extract provided a significant protective effect against the irradiation.

It is reported that ionizing radiation increasing IL-1 $\beta$  production and oxidative stress reaction negatively impacts human health <sup>(3,4)</sup>. It is also reported that Ionizing radiation activates JNK to promote the generation of ROS via Nox1 expression, and these ROS are involved in the formation of Ionizing radiation-induced micronucleus <sup>(15)</sup>. IL-1 $\beta$  is a central mediator of inflammatory responses, secretion of IL-1 $\beta$  is associated with inflammasome in inflammatory cells <sup>(5,6)</sup>. There are several kinds of inflammation known to contain NOD-like receptors (NLRs) and PYHIN proteins, such as NLRP1, NLRP3, NLRC4 and AIM2 <sup>(16,17)</sup>.

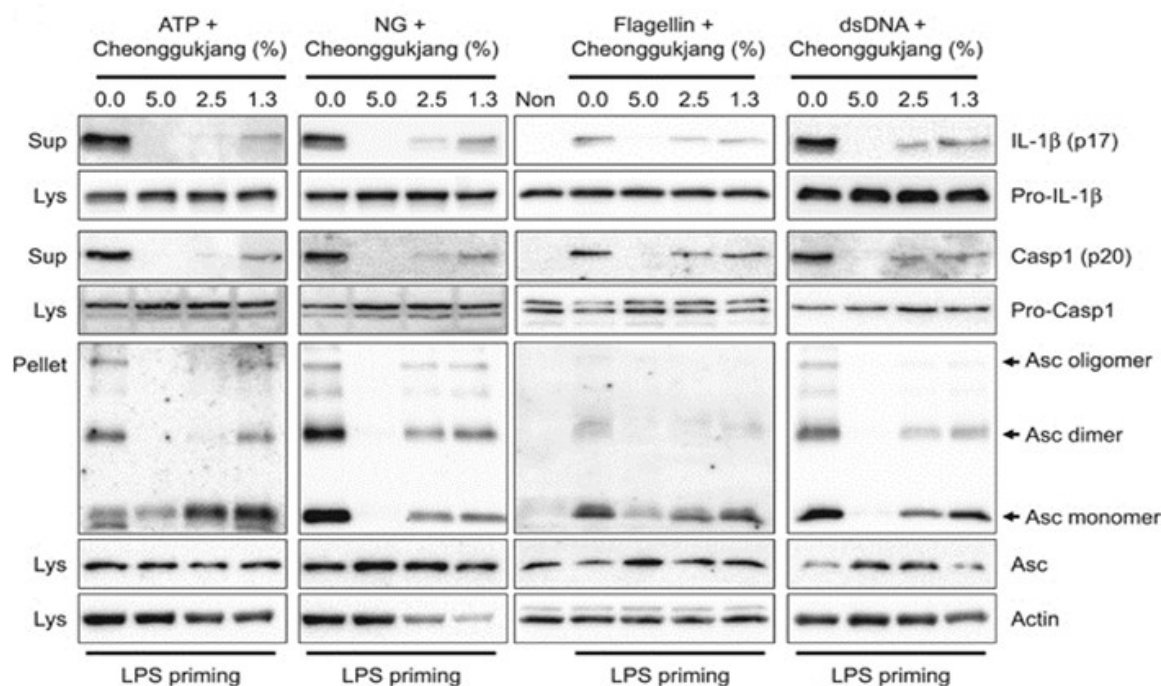
The NLRP3 inflammasome is currently the most fully characterized inflammasome and consists of the NLRP3 scaffold, the apoptosis-associated speck-like protein containing a CARD domain (ASC) adaptor, and caspase-1 <sup>(16)</sup>. The NLRP3 inflammasome is a cytoplasmic protein complex that mediates inflammatory responses to a broad array of danger signals. Pathogen-associated and damage-associated molecular pattern molecules and environmental irritants can activate NLRP3. Through the ASC adaptor protein, NLRP3 recruits and activates caspase-1, leading to the cleavage of the pro-inflammatory cytokine IL-1 $\beta$  and IL-18 precursors <sup>(17)</sup>. NLRP3 inflammasome activation and subsequent IL-1 $\beta$  formation have been shown to contribute to radiation radiation-induced micronucleus formation <sup>(18)</sup>. Furthermore, the NLRP3 inflammasome promoted radiation-induced pulmonary inflammation by significantly upregulated caspase-1, IL-1 $\beta$  <sup>(19)</sup>. The AIM2 composed of a pyrin domain to recruit ASC and a DNA-binding HIN domain, AIM2 inflammasome activation

induced the maturation of pro-IL-1 $\beta$  to IL-1 $\beta$  by double-strand DNA <sup>(16)</sup>. Irradiation triggered the AIM2 and IL-1 $\beta$  upregulation contributed to the radiation pneumonitis <sup>(20)</sup>.

In addition, we observed that CGJ extract attenuated inflammasome activation by its activators such as ATP and nigericin for NLRP3, flagellin for NLRC4 and dsDNA for AIM2 in published results (figure 4) <sup>(13)</sup>. Inflammasome activation involves the production of activated caspase-1 (p10 and p20) and the induction of pyroptosis. We observed the effects of the CGJ extract on the secretion of caspase-1 (p20) in the cell supernatant and on the formation of the ASC pyroptosome in the insoluble pellet after cross-linking with disuccinimidyl suberate. CGJ extract reduced the formation of the ASC pyroptosome by the inflammasome activators. Thus, the CGJ extract inhibited caspase-1 secretion and ASC pyroptosis preceding inflammasome activation, similar to its effects on IL-1 $\beta$  secretion. Taken together, the results show that the CGJ extract attenuated the secretion of IL-1 $\beta$  and caspase-1, as well as ASC pyroptosome caused by NLRP3, NLRC4 and AIM2 inflammasome activation.

CGJ extract inhibited secretion IL-1 $\beta$  or casepace-1 via attenuation of inflammasome activation. CGJ extract also inhibited radiation-induced micronucleus formation in the mouse model. It appears that CGJ extract acts as a potent anti-inflammatory agent both *in vitro* and *in vivo*. The CGJ extract could suppress inflammation and chromosomal aberrations through more than one type of mechanism, depending on the type of pathogen-associated or damage-associated molecular pattern molecules.

Base on the discussion above, we conclude that CGJ extract attenuates radiation-induced micronucleus formation possibly mediated by inhibiting NLRP3 or AIM2 and another inflammasome. The mechanisms of effect of CGJ extract will provide valuable information in the future studies.



**Figure 4.** Effects of CGJ extract on caspase-1 activation and the ASC pyroptosome [http://www.nature.com/cmi/journal/vaop/ncurrent/fig\\_tab/cmi201613f1.html](http://www.nature.com/cmi/journal/vaop/ncurrent/fig_tab/cmi201613f1.html). LPS-primed BMDMs were treated with ATP (2mM), NG (40μM), flagellin (0.5mg/ml) or dsDNA (1μg/ml) for 1h in the presence of the indicated cheonggukjang concentration.

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**Conflicts of interest:** Declared none.

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