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RESEARCH ARTICLE Indian Journal of Animal Research

In vitro Study on the Immunomodulatory and Anti-inflammatory Effect of Soregen® Technology Water in Broiler and Layer Hens Peripheral Blood Mononuclear Cells

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10.18805/IJAR.BF-1798

ABSTRACT

Background: Peripheral blood mononuclear cells (PBMCs) represent an attractive tissue source in pharmacogenomics and molecular and immunologic studies. In order to investigate the changes in biological effects, water samples were exposed to a specific quantum entanglement signal from the SoRegen chip for 48 hours. This study aimed to evaluate the immunomodulatory and anti-inflammatory effects of SoRegen Technology in broiler and layer hens PBMCs.

Methods: Six-week-old Arbor Acress broiler chickens and 66-week-old layer hens Lohmann were used for the establishment of an *in vitro* cell culture model with chicken PBMCs.

Result: The result indicates that post-treated water media enhanced the proliferative properties of broiler and layer hens PBMCs. The mRNA and protein expression of immune-modulating and pro-inflammatory cytokines: IL-2, IL-6, iNOS, IFNγ, TNFα and NFκB exhibited a marked increase following stimulation of the cells by LPS and Con A when compared with the non-stimulated cells. However, stimulated cells grown in structured water media showed a strikingly decreased expression of pro-inflammatory cytokines, except for the upregulation of IFNγ expression in broiler PBMCs but not in layer hens PBMCs. It can be concluded that SoRegen® Technology water had anti-inflammatory activities with potential clinical immunomodulatory effects in younger chicks.

Key words: Anti-inflammatory, Immunology, PBMCs, SoRegen® Technology.

INTRODUCTION

Controlled *in vitro* systems using freshly isolated immune cells from blood can be used to investigate the mode of action of immunomodulatory compounds such as drugs or feed additives and represent a promising alternative to animal experiments. Cytokine expression varies and this expression plays a critical role in the modulation of immune responses in the host (Varughese *et al*., 2019) and have pleiotropic effects on different cells (Kany *et al*., 2019). Peripheral Blood Mononuclear Cells (PBMCs) are an important class of immune cells and play a critical role in the activation of both innate and adaptive immune responses. The PBMCs of animals can be primarily cultured and used as a nutritional development strategy and can contribute to the prevention of infectious diseases and further improve animal health (Larsberg *et al*., 2021). Most of PBMCs comprise 70-90% lymphocytes, 10-20% monocytes, 1-2% dendritic cells and a trace amount of circulating stem cells including erythroid progenitors (Zhang *et al*., 2020). They act as the major source for different cytokines, which have a major role in immune responses in the host (Takeuchi and Akira, 2010). PBMCs have been useful in studying the immune response (Gali *et al*., 2024).

Nowadays there is a quite different new and unique methodology to improve the characteristics of the material. Especially in recent years, with the interdisciplinary development of quantum informatics, physical properties and measurement of quantum entanglement have been investigated and analyzed. Entangled state theory plays an important role in the transmission of quantum mechanical Information (Zou, 2021). SoRegen technology (ST) proposed that exposure to a specific quantum entanglement signal can transfer information that may cause change and improve the function of the target material. Quantum entanglement is the state where two systems are so strongly correlated that gaining information about one system will give

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How to cite this article: Purnamasari, L., dela Cruz, J.F., Lee, D.B., Choi, Y.J., Yi, J.K. and Hwang, S.G. (2024). *In vitro* Study on the Immunomodulatory and Anti-inflammatory Effect of Soregen® Technology Water in Broiler and Layer Hens Peripheral Blood Mononuclear Cells. Indian Journal of Animal Research. DOI:10.18805/IJAR.BF-1798.

Submitted: 04-04-2024 **Accepted:** 25-06-2024 **Online:** 26-07-2024

immediate information about the other no matter how far apart these systems are (Jeffrey, 2020). Although these entangled particles are not physically connected, they still are able to share information instantaneously (Herbst *et al*., 2015; Zou, 2021). A similar phenomenon was observed by (Hwang *et al*., 2017) of structured water composed of an exclusive zone by utilizing far-infrared ray waves method which exhibits antioxidant properties with increased cellular bioactivity and can be an active constituent in cell biology.

One theory suggests that water molecules have the ability to form unique structures and rearrange themselves and store the information within their molecular framework based on their environment. This transfer could occur through direct contact, through electromagnetic fields and frequencies (Beauvais, 2021). Water structures can hold information and retain it over time (Absalan *et al*., 2021). In order to investigate the changes in biological effects, water samples were exposed to a specific quantum entanglement signal from the SoRegen chip for 48 hours. This study evaluates the immunomodulatory and anti-inflammatory effects of SoRegen Technology in broiler and layer hens PBMCs.

MATERIALS AND METHODS

The experiment was conducted in June 2022 at the Applied Biochemistry Laboratory, Hankyong National University, South Korea.

Isolation of PBMCs using Histopaque-1077

Six-week-old Arbor Acres broiler chickens and 66-week-old layer hens chicken Lohmann from three birds in each breed were used to establish an *in vitro* cell culture model with chicken PBMCs. The blood was collected from the brachial wing vein in ethylene diamine tetra acetic acid tubes containing a K3EDTA anticoagulant. PBMCs were isolated using Histopaque-1077 (Sigma-Aldrich) as previously described by (Annamalai *et al*., 2014) with modification. The blood samples were diluted with 2-4 volumes phosphate buffer saline (PBS, Gibco) pH 7.2 then layered over ficoll Histopaque with a density of 1077 g/ml in equal volumes and centrifuged at 400×g for 45 min at 20°C in swinging bucket rotor without brake to separate cell-poor plasma from the blood-cell fraction and three layer hens could be observed. The top of the erythrocyte layer hens containing the PBMCs was carefully collected in a 50 mL tube and then centrifugated at 300×g for 30 min at 20°C. The buffer-layer hens containing the PBMCs were collected, washed twice and centrifuged at 200×g for 15 min at 20°C. After centrifugation, cells were resuspended in a 10 mL RPMI-1640 medium (Gibco) containing 10% fetal Bovine Serum (FBS, Biowest), 1% penicillin/streptomycin (Lonza) and incubated at 37°C under 5% CO_2. The medium will be replaced every 2 days.

SoRegen water treatment

Commercial water (Sam Da Soo Jeju mineral water) samples were exposed to a specific quantum entanglement signal from the SoRegen chip for 48 hours (post-exposure water). A special information programming chip (Chip A for improving animal health) was used from the information antenna to expose information to the water via a zoning chamber (Fig 1). Molecular structure changes through electron rearrangement by receiving the information wave field signal inside the chamber and resonating the information to the water to cause the quantum entanglement phenomena. The water is then used for making a cell growth media (Triple distilled water (TW) for Control, pre-exposure water (commercial water) and post-exposure water. The cells were grown in different media to investigate the immunomodulatory and anti-inflammatory action in chicken PBMCs. To make the media, RPMI powder was dissolved in 890ml water, 3.7 g sodium bicarbonate ($NaHCO_3$, VWR Life Science) was added and pH was adjusted to 7.3 with 0.2N HCl (Samchun). Cells were grown in RPMI media with 10% FBS supplemented with 1% Penicillin-Streptomycin and incubated at 37°C under 5% CO_2.

Cell viability assay

For the cell proliferation experiment, cells were seeded in a 96-well plate, 100 µl/well at a density of $(1\times10)^5$ per ml with 8 replications and incubated for 6 hours at 37°C under 5% CO_2. After the cells were attached, they were treated with different media preparations (control, pre-exposure water and post-exposure water) for 24- and 48-hours incubation at 37°C under 5% CO_2. After each time frame, the media was removed and 100 µl/well fresh media was added with 10 µl/well of Quanti-MAX™ WST-8 Cell Viability Assay Kit (Biomax) and again incubated for 2-4 hours at 37°C under 5% CO_2 to quantify cell proliferation activity.

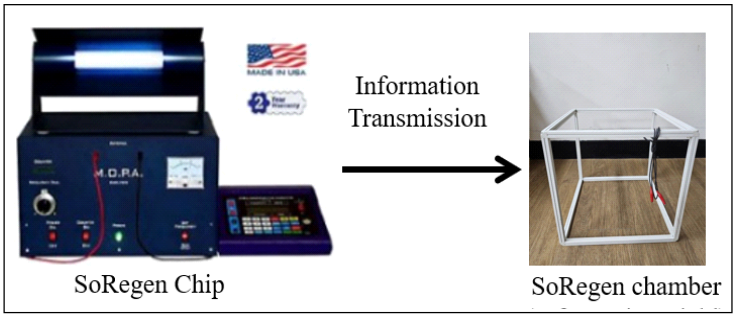


Fig 1: Information transmission procedure.

Optical Density was measured under 450 nm using a Microplate Reader, Tecan Infinite F50 Instrument. The percentage of cell proliferation was computed concerning the control.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

For the RT-PCR, PBMCs were seeded in a 6-well plate, 2 ml/well at a density of 5×10^5 per ml and incubated for 3 hours at 37°C under 5% CO_2. Cells were then treated with different media preparations. To activate macrophages, 1 µg/ml of lipopolysaccharide (LPS, Sigma-Aldrich) and 5 µg/ml Concanavalin A (Con A, Sigma-Aldrich) were added. PBMCs were harvested from individual wells in each experiment (biological replicate). The concentration and purity of RNA isolates were measured with a NanoDrop 2000 spectrophotometer (Scientific Nanodrop Products, Wilmington, NC, USA). Total cellular RNA was extracted using Trizol reagent (Molecular Research Center, Inc). cDNA was synthesized from the 1µl of total RNA sample in a 20 µl RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) containing 10 mM dNTP Mix, 0.2 µg/ml random hexamer primer, 40 U/µl Riboblock RNAse inhibitor, 200 U/µl RevertAid Reverse Transcriptase and 5× reaction buffer. PCR reactions consisted of an initial denaturation cycle at 95°C for 5 min, followed by 30 amplification cycles: 40 seconds at 95°C, annealing for 40 sec with range temperature 58-62°C and extension at 72°C for 1 min. Specific primers were used to amplify the genes related to inflammatory and immune function. PCR products were then separated by electrophoresis using 2% agarose with ethidium bromide and transillumination was done to capture the mRNA bands. ImageJ software was used to measure the quantify bands obtained from agarose gel electrophoresis. Primer sequences are shown in Table 1. based on research by (Wandita *et al*., 2018) with modification.

Western blot

For the western blot, PBMCs were seeded in a 6-well plate, 2 ml/well at a density of 5×10^5 per ml and incubated for 3 hours at 37°C under 5% CO_2. Cells were then treated with different media preparations. To activate macrophages, 1 ug/ml of LPS and 5 ug/ml of Con A were added. Protein was extracted from PBMCs by adding 200 µL of protein extraction solution (iNtRON Biotechnology) and then transferred from 6-well plates to 1.5 mL tubes. The lysate was purified by centrifugation at 15000 rpm for 15 minutes 4°C and protein concentrations were determined using Bradford assay. Diluted 30 µg of the protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose transfer membranes using Mini Trans-Blot® Cell and Criterion™ Blotter (Bio-Rad). The membranes were blocked with 5% skimmed milk and hybridized with specific primary polyclonal antibodies to identify different proteins (IL-2, IL-6, iNOS, IFNγ, TNFα and NFκB) related to inflammatory and immune function (Abcam). Specific proteins were identified by further incubation of the corresponding membranes with horseradish peroxidase-conjugated (HRP, Abcam) secondary antibodies followed by treatment with enhanced chemiluminescence (AB Frontier). The target proteins were exposed and detected in radiographic films. ImageJ software was used to measure the quantified bands obtained from radiographic films of western blot.

Statistical analysis

The result was expressed as the mean±standard deviation. Comparisons were based on one-way ANOVA followed by Duncan's multiple range test, $P<0.05$ using IBM SPSS statistics 24.

RESULTS AND DISCUSSION

SoRegen Technology water was evaluated for its effect on the proliferation of broiler and layer hens PBMC (Fig 2). Results show that the proliferation of cells in a culture media prepared with post-exposure water was significantly higher ($P<0.05$) compared to the control and pre-treated water after growing for 24 and 48 hours. The data showed that post-exposure water media enhanced the proliferative properties of broiler PBMCs. The result also indicates that no negative effect was observed on the viability of cells grown in post-exposure water media.

The structured water phenomenon that is composed of an exclusive zone exhibits antioxidant properties with increased cellular bioactivity and can be an active

Table 1: Sequence of primers used in RT-PCR for broiler and layer hens PBMCs.

Gene	Primer sequence (5′-3′)		Amplicon size
	Forward	Reverse	(bp)
β-actin	CCCCCGTGCTGTGTTCCCAT	GGGTGCTCCTCAGGGGCTAC	271
IL-2	TCT GGG ACC ACT GTA TGC TCT	ACA CCA GTG GGA AAC AGT ATC A	270
IL-6	CAA GGT GAC GGA GGA GGA C	TGG CGA GGA GGG ATT TCT	123
iNOS	ATT CCA AAC ATC CTG GAG GTG	TCA TAG AGA CGC TGC TGC CAG	371
IFNγ	GAT GAC TTG CCA GAC TTA CAA C	AGC AAT TGC ATC TCC TCT GAG ACT G	403
TNF-α	CAT CTT CTC AAA ATT CGA GTG ACA A	TGG GAG TAG ACA AGG TAC AAC CC	184
NFκB	GGC CTG CAA AGG TTA TCG TT	TGT CTG TGA GTT GCC GGT CT	124

IL-2= Interleukin-2, IL6= Interleukin-2; iNOS= Inducible no synthase; IFNγ= Interferon γ; TNF-α= Tumor necrosis factor-α; NFκB= Nuclear factor-κB.

constituent in cell biology (Hwang *et al*., 2017). In layer hens PBMC (Fig 2) a faster effect on the proliferation was observed. PBMC cultured in post-exposure water media has a significantly higher percentage of proliferation compared to the other groups in both 24 and 48-hour time frames. This shows that layer hens PBMCs are more responsive to changes in their culture media as compared with the broiler PBMCs. However, it is also good to note that at 48 hours, the proliferation of broiler PBMC grew in post-exposure water is higher compared to pre-exposure water cultured PBMCs.

There has been an argument regarding the physicochemical properties of structured water and the possibility that it may influence biological activities depending on the hydrophobic or hydrophilic properties of the cell membrane and other organic components of cells. It has been reported by (Hwang *et al*., 2017; Ives *et al*., 2014) that the increase of the ultra-weak photon emission is associated with an oxidation reaction. The number of photon emissions is expected to decrease for water with an antioxidant property. These antioxidant properties inhibit or delay the oxidation of other molecules such as lipids, proteins, or other molecular components in cells by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu *et al*., 1998). This is also called as quantum entanglement phenomenon. Quantum entanglement gaining information about one system will give immediate information about the other no matter how far apart these systems are (Jeffrey, 2020). Although these entangled particles are not physically connected, they still are able to share information instantaneously (Herbst *et al*., 2015; Zou, 2021). SoRegen Technology can enable new properties to emerge from a specific material on the water. Water molecules/structures rearrange and store the information within their molecular framework based on transfer electromagnetic fields (Absalan *et al*., 2021; Beauvais, 2021).

Antibody response in layer-type birds is stronger and longer lasting than in broilers, making layer hens more suited for long-term humoral immune response (Koenen *et al*., 2002). Therefore, a difference in the metabolic potential of immune cells between these lines likely reflects their vastly different production traits and the effects on immune system resources. Study by (Meyer *et al*., 2022) showed that commercial broiler and layer hens PBMCs were metabolically challenged using carbonyl cyanide-4 phenylhydrazone and oligomycin. However, layer hens cells showed a numerically higher. Layer hens maintain significant physical activity throughout the production (Kozak *et al*., 2016). Broiler selection for weight gain has decreased immune system resources due to the behavior in part to energy allocation toward significant weight gain that takes away from metabolic costs of standing, walking, perching, *etc*. (Tickle *et al*., 2018). Reactivity of immune system differences between layer hens and broilers arise from the difference in lifespans.

To better understand the inhibitory effects of SoRegen Technology water on inflammation, mRNA (Fig 3 and 4) and protein expression (Fig 5 and 6) of immune modulating and pro-inflammatory cytokines: IL-2, IL-6, iNOS, IFNγ, TNFα and NFκB were investigated *via* RT-PCR and Western Blot in broiler and layer hens PBMCs stimulated with LPS and Con A. The mRNA and protein expression of immune-modulating and pro-inflammatory cytokines: IL-2, IL-6, iNOS, IFNγ, TNFα and NFκB exhibited a marked increase following stimulation of the cells by LPS and Con A when compared with the non-stimulated cells ($P<0.05$). However, stimulated cells grown in post-exposure water media showed a strikingly decreased expression of pro-inflammatory cytokines, except for the upregulation of IFNg expression in the broiler PBMCs ($P<0.05$). However, expression of pro-inflammatory cytokines in layer hens PBMCs decreased only on iNOS and NFκB ($P<0.05$).

Inhibition and mutation of iNOS have resulted in diminished immunological response against intracellular pathogens (Salim *et al*., 2016). It is well known that the overproduction of NO by iNOS plays a critical role in the regulation of the inflammatory process. RT-PCR and Western Blot analysis of broiler and layer hens PBMCs showed clear inhibition in the expression of iNOS, which suggests that the structured water may induce the reduction

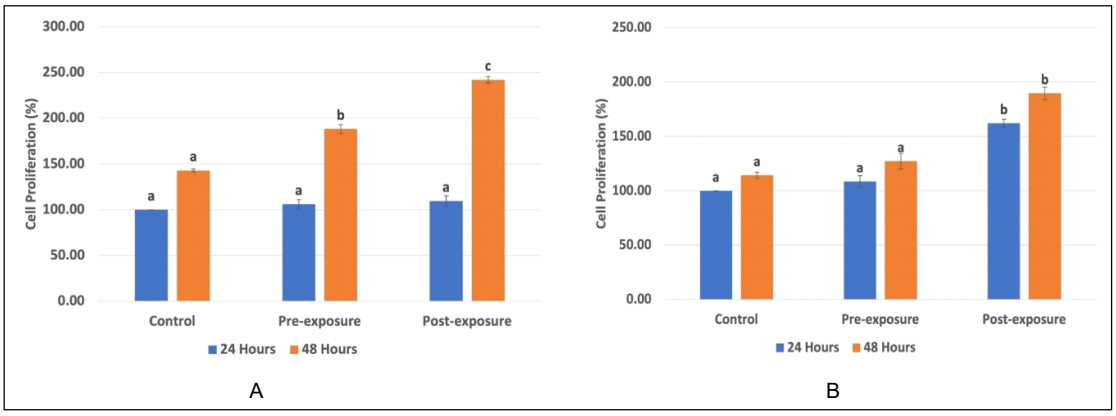


Fig 2: Effect of SoRegen Technology water on the proliferation of (A) broiler and (B) layer hens PBMC.

of NO production by the transcriptional suppression of the iNOS gene.

Interleukin-6 plays an important role in autoimmune diseases, bacterial infections and metabolic side effects (Rose-John, 2018). IL-6 activation has a pivotal role in inflammation and inflammation-induced cell damage. In chickens, it was recently reported that the chicken IL-6 (chIL-6) gene was cloned from the chicken macrophage-like cell line HD11 and recombinant chIL-6 (rchIL-6) was generated (Nishimichi *et al*., 2005). The sequence identities for human and avian interleukins are only 31 to 35% (Chen *et al*., 2016). Hence, IL-6 modulation has been used as a target for anti-inflammatory drug development (Tanaka *et al*., 2014) and probiotics (Putra *et al*., 2023). LPS stimulation increases the expression of inflammatory cytokine IL-6 suppressing growth processes by directing nutrients away from body protein accretion to support the immune response (Tan *et al*., 2014). In the present study, broiler PBMCs cultured in structured water reduced the mRNA and protein expression level of proinflammatory cytokine IL-6. This might be due to the anti-inflammatory potential of structured water through downregulating NFκB signaling pathway by

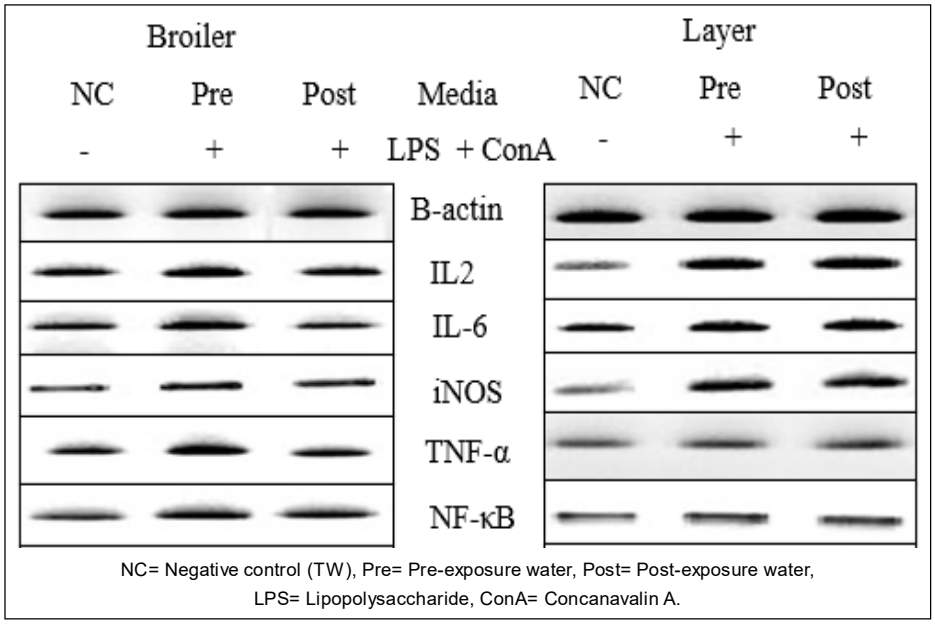


Fig 3: Effect of SoRegen technology water on the mRNA expression of inflammatory and immunomodulatory genes in (A) Broiler and (B) Layer hens PBMC.

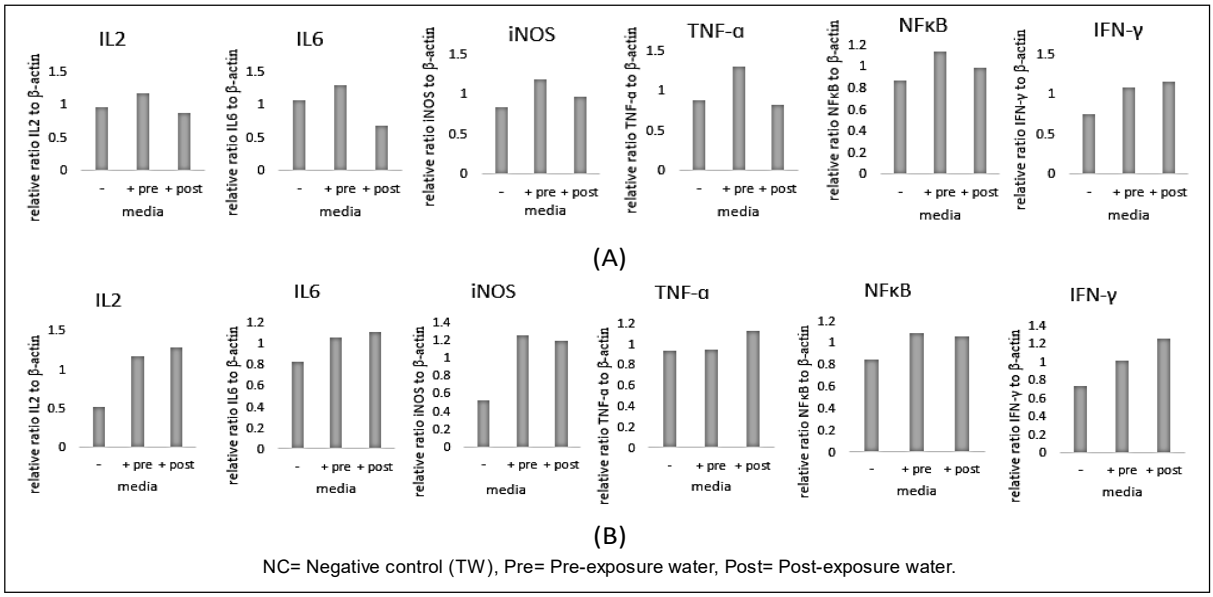
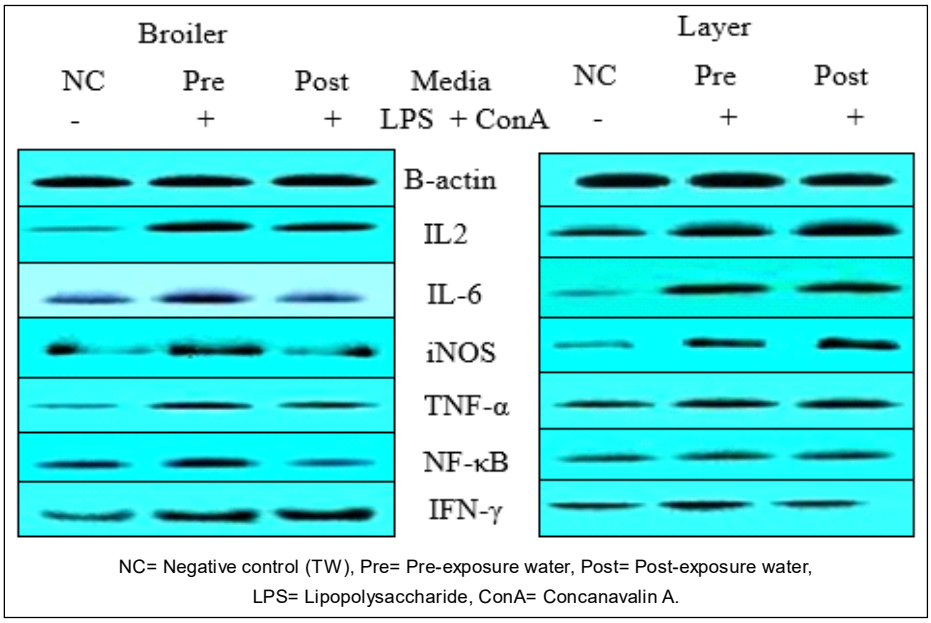


Fig 4: Effect of SoRegen Technology water on the relative ratio of mRNA expression of inflammatory and immunomodulatory genes in (A) Broiler and (B) Layer hens PBMC.

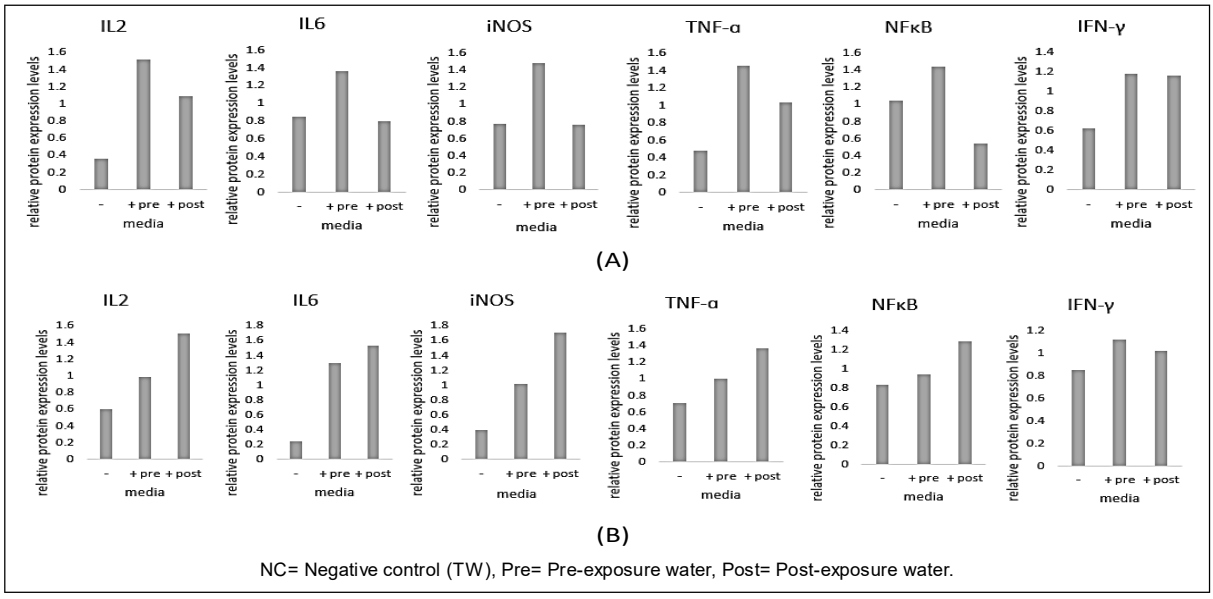
decreased phosphorylation of NFκB (Boyman and Sprent, 2012).

NFκB controls various stages of inflammation and immune modulation via the regulation of several molecules, including cytokines (IL-1β, TNF-α), iNOS and chemokines in rats (Wei *et al*., 2015) and IL-1β, IL-6, IL-18 and IFN-γ in chicken B cells (Gupta *et al*., 2013). Proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, which play crucial roles in the development of inflammatory diseases, are also involved in innate immunity and autoimmune diseases (Liu *et al*., 2017). Thus, blocking the effects of proinflammatory mediators offers an attractive therapeutic strategy. Moreover, post-exposure water stimulates the production of IL-2 and IL-6 in layer hens PBMCs, activates chicken macrophages to produce inflammatory mediators which are produced by activating T helper cells 1 (Th1) and plays a central role in cell-mediated immunity (Gupta *et al*., 2013). This suggests that post-exposure water exerts immunomodulatory effects in layer hens through mediating both cellular and humoral immunity. TNF-α is a pro-inflammatory cytokine that was clearly upregulated by the stimulation with LPS and Con A. LPS treatment also



NC= Negative control (TW), Pre= Pre-exposure water, Post= Post-exposure water, LPS= Lipopolysaccharide, ConA= Concanavalin A.

Fig 5: Effect of SoRegen technology water on the protein expression of inflammatory and immunomodulatory genes in (A) Broiler and (B) Layer hens PBMC.



NC= Negative control (TW), Pre= Pre-exposure water, Post= Post-exposure water.

Fig 6: Effect of SoRegen Technology water on densitometric value of protein expression of inflammatory and immunomodulatory genes in (A) Broiler and (B) Layer hens PBMC.

induces production of nitrite in avian macrophages (He *et al*., 2011). In the present study, this upregulation was alleviated in the cells cultured with post-exposure water, indicating that ST-water may benefit the attenuation of inflammatory response stimulated by LPS and Con A.

Interferon-γ (INFg), is a pro-inflammatory cytokine mainly produced by T helper type 1 cells, CD $\alpha 8^{+}$ cytotoxic lymphocytes, natural killer cells, B cells, natural killer T cells and professional antigen-presenting cells (Bagheri *et al*., 2022). IFN-γ plays a vital role in innate and adaptive immunity, activating the host's defense against viral and bacterial infection. IFN-γ exerts its immunological function by activating various host cells, especially monocytes and macrophages and also can regulate the expression of class II major histocompatibility complex by releasing pro-inflammatory and inflammatory cytokines (Yuan *et al*., 2018). As professional phagocytic cells, monocytes and macrophages are indispensable in the host defense mechanism, enhancing the immune response by releasing pro-inflammatory and inflammatory cytokines. The upregulation of IFNγ in broiler and layer hens PBMCs cultured in ST-water shows the possible function of structured water in immunomodulation.

CONCLUSION

These data demonstrated that ST-exposed water modulated the immune and inflammatory response of broiler and layer hens PBMCs. Further research on oral administration of Soregen Technology water as drinking water in living animals must be conducted to increase the validity of this experiment and the possibility of improving the immune status of chickens to resist diseases.

ACKNOWLEDGEMENT

The research was supported and funded by the Research Council of SoRegen Convergence Science, Seoul, Republic of Korea.

Author's contribution

L. Purnamasari, J.F. dela Cruz, D.B. Lee and SG. Hwang conceived and designed the experiments. L. Purnamasari and J.F. dela Cruz performed the experiments. All authors analysed the data. L. Purnamasari and J.F. dela Cruz writing of the manuscript. All authors critically revised the manuscript and approved the final version.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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