Original Article

Walnut polyphenol extracts inhibit *Helicobacter pylori*-induced STAT3^{Tyr705} phosphorylation through activation of PPAR-γ and SOCS1 induction

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The health beneficial effects of walnut plentiful of *n*-3 polyunsaturated fatty acid had been attributed to its anti-inflammatory and anti-oxidative properties against various clinical diseases. Since we have published *Fat*-1 transgenic mice overexpressing *3-desaturase* significantly mitigated *Helicobacter pylori* (*H. pylori*)-associated gastric pathologies including rejuvenation of chronic atrophic gastritis and prevention of gastric cancer, in this study, we have explored the underlying molecular mechanisms of walnut against *H. pylori* infection. Fresh walnut polyphenol extracts (WPE) were found to suppress the phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) induced by *H. pylori* infection in RGM-1 gastric mucosal cells. Notably, *H. pylori* infection significantly decreased suppressor of cytokine signaling 1 (SOCS1), but WPE induced expression of SOCS1, by which the suppressive effect of walnut extracts on STAT3^{Tyr705} phosphorylation was not seen in SOCS1 KO cells. WPE induced significantly increased nuclear translocation nuclear translocation of PPAR-γ in RGM1 cells, by which PPAR-γ KO inhibited transcription of SOCS1 and suppressive effect of WPE on p-STAT3^{Tyr705} was not seen. WPE inhibited the expression of c-*Myc* and IL-6/IL-6R signaling, which was attenuated in the RGM1 cells harboring SOCS1 specific siRNA. Conclusively, WPE inhibits *H. pylori*-induced STAT3 phosphorylation in a PPAR-γ and SOCS1-dependent manner.

***Key Words*: *Helicobacter pylori*, walnut polyphenol extract, STAT3^{Tyr705}, SOCS1, PPAR-γ**

Helicobacter pylori (*H. pylori*), a gram-negative bacterial pathogen living in stomach, has been implicated in development of gastric cancer through the development of precancerous lesions such as hypertrophic and atrophic gastritis, responsible for initiating and progressing mechanism implicated in gastric carcinogenesis.[1] Among mechanisms in *H. pylori*-carcinogenesis, including NF-κB based oxidative stress, cytokines mediated mutagenesis, apoptosis induced atrophic changes, and more carcinogenic actions,[2,3] it has been well documented as molecular pathomechanism that *H. pylori* infection causes the activation of signal transducer and activator of transcription 3 (STAT3), STAT3 phosphorylated on Tyr705 residue (p-STAT3^{Tyr705}), forms a dimer, translocates to nucleus, and functions as transcription factor to regulate the target genes implicated in *H. pylori*-associated inflammation and carcinogenesis subsequent to IL-6 activation.[4–7] Though STAT3^{Ser727} phosphorylation can be pathogenically involved in oxidative stress and malignant transformation relevant to *H. pylori* infection since STAT3^{Ser727} localizes in mitochondria and associates with *Ras* dependent oncogenic transformation, p-STAT3^{Tyr705} had been revealed to be implicated in either Barrett's esophagus and *H. pylori*-associated oncogenic inflammation.[8,9] *Cag*A cytotoxin from *H. pylori* led to significant cytokine signaling pathways via MAPK activation and is responsible for gastric inflammation and carcinogenesis including increased cell proliferation, angiogenesis, inflammation, inhibition of immunocytes, and epithelial cell apoptosis.[10] Among these cytokines, IL-6, IL-6R, and gp130 with subsequent STAT3 activation are representationally dysregulated pathways in *H. pylori* infection.[11]

Therefore, agents or intervention to regulate IL-6 signaling seem to be of potential significance to solve unmet medical needs of *H. pylori*-associated gastric pathologies. Suppressor of cytokine signaling (SOCS) has been known to cope with oxidative stress and inflammatory cytokines relevant to *H. pylori* infection,[12,13] especially SOCS 1 that SOCS1,[14] named STAT induced STAT inhibitor (SSI) or JAK-binding protein (JAB), played immune regulation as well as inflammatory modulation, leaving the agents or drug having the potential of autoimmunity as well as cancer.[15,16]

Supported with our previous publications that *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) generating *fat*-1 transgenic mice showed significant rescuing from *H. pylori*-associated atrophic gastritis as well as gastric cancer,[17–19] we put hypothesis that walnut polyphenol extracts (WPE) containing abundant *n*-3 PUFAs can be food factor to ameliorate *H. pylori*-associated gastric inflammation and executed the current experiment to explore molecular mechanisms to limit *H. pylori*-associated IL-6 and their STAT3 activation before *in vivo* model of *H. pylori*-associated gastritis.

Materials and Methods

Materials. RPMI-1640 medium, fetal bovine serum, penicillin (FBS), streptomycin were products of GIBCO BRL (Grand Island, NY) and materials for culturing *H. pylori* were sheep blood agar,

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Gaspak and anaerobic jars (BD Biosciences, Sparks, MD). Primary antibody against actin was purchased from Sigma-Aldrich Co. (St. Louis, MO), antibodies for lamin B from Santa Cruz Biotechnology (Santa Cruz, CA), other antibodies for p-STAT3^{Tyr705}, total STAT3 from Cell Signaling Technology (Beverly, MA), horseradish peroxidase (HRP)-conjugated secondary antibody from Pierce Biotechnology (Rockford, IL). DL-dithiothreitol (DTT), TRIzol, 4',6-diamidino-2-phenylindole (DAPI) from Invitrogen (Carlsbad, CA), and polyvinylidene difluoride (PVDF) membranes were supplied from Gelman laboratory (Ann Arbor, MI). The ECL chemiluminescent detection kit was purchased from LPS solution (Daejon, South Korea) and protein assay dye (Bradford) reagent was supplied by Bio-Rad Laboratories (Hercules, CA), bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Biotechnology.

Preparation of WPE. Walnut polyphenol extract (WPE) from English walnuts (*J. regia*, California Walnut Commission) was prepared according to a previously described methanolic extraction method.[20] Briefly, after the walnuts were frozen for 24 h, the shelled kernels were finely ground and immersed in a solution of 75% acetone containing 526 μm/L sodium metabisulfite. The solution was subsequently purged with N_2 to prevent oxidation and was incubated at 4°C. After 24 h, the solution was decanted, thereby resulting in a cold extract that was centrifuged at 8,000 × *g* for 10 min. The resulting supernatant was filtered using Whatman filter paper No. 2. To remove lipids from the sample, the acetone was removed under reduced pressure and methanol (50% aqueous, v/v) was added. After three consecutive hexane extractions, the extracts were lyophilized to a dry powder after removing the methanol to prevent oxidation. All of the prepared samples were stored at 80°C until needed.

Cell culture. RGM-1 cells from Prof. Hirofumi Matsui (Tsukuba Univ, Japan) were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in an incubator with humidified atmosphere of 95% O_2/5% CO_2.

Bacteria strain and infection condition. *H. pylori* (ATCC 43504) with the typical S shape, gram-negative rods, possessing the *Cag*A and *Vac*A were provided in a frozen state by ATCC. *H. pylori* ATCC 43504 strains were grown on tryptic soy agar with 5% sheep blood agar (BD Diagnostics) and Dent antibiotics supplement (Oxoid) at 37°C under microaerophilic conditions (Campy-Pak 273 System, BBL). RGM1 cells were incubated overnight in fresh serum- and antibiotic-free RPMI 274 medium and were infected with *H. pylori* at multiplicities of infection (MOI) of 50:1.

Western blot analysis. The cell lysates were prepared, and the protein concentration was measured as described previously 7. The equivalent amounts of proteins (10–30 μg) were subjected to electrophoresis on 8% or 12% SDS-polyacrylamide gel and transferred to PVDF membrane. The transferred proteins were blocked in 5% fat-free dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) for 1 h at room temperature. Then, the membranes were incubated with primary antibodies in 3% fat-free dry milk in PBS overnight in 4°C. Membranes were washed followed by incubation with 1:3,000 dilution of respective HRP conjugated secondary antibodies for 1.5 h and again washed with PBST. Protein expressed was visualized with an ECL chemiluminescence detection kit.

Preparation of cytosolic and nuclear extracts. After *H. pylori* infection, cells were washed twice with ice-cold 1× PBS and scraped in 1 ml of PBS, followed by centrifugation at 1,700 × *g* for 5 min at 4°C. Pellets were resuspended in hypotonic buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonylfluoride (PMSF)] for 15 min on ice. Ten % Nonidet P-40 was then added to final concentration of 0.1% for less 3 than 5 min. The mixture was then centrifuged at 6,000 × *g* for 5 min at 4°C. Supernatant was collected as the cytosolic extract and stored at –80°C. The pellets were washed twice with hypotonic buffer A and resuspended again in hypertonic buffer C [20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM DTT and 0.2 mM PMSF] for 1 h on ice and centrifuged at 18,000 × *g* for 15 min at 4°C. The supernatant containing nuclear proteins was collected and stored at –80°C. The protein concentrations of both fractions were determined by using the BCA protein assay reagent.

Confocal imaging analysis. Cells were infected with *H. pylori* for 12 h. After the cells were incubated with probe (1 nM) in prewarmed staining solution for 30 min, samples were fixed with cold 95% MeOH/5% acetic acid for 10 min at 4°C. Then samples were permeabilized with 0.2% Triton X-100 for 5 min at room temperature and blocked with 5% bovine serum albumin in PBST for 1 h at room temperature. Samples were incubated with primary antibody specific for phospho-STAT3^{Tyr705} overnight at 4°C, followed by incubation with fluorescein isothiocyanate-goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Nuclear-staining was performed with DAPI for 5 min at room temperature. Images were assessed under a fluorescent microscopy.

Statistical analysis. Data from three independent experiments at least were expressed as the mean ± SD. The statistical significance of differences between two groups was evaluated using Student's *t* test. Analysis was performed using Sigma plot (ver. 10). Statistical significance was accepted at $p<0.05$, unless otherwise indicated.

Results

Walnut extract inhibited *H. pylori*-induced IL-6 and additional inflammatory mediators including COX-2, c-*Myc*, tumor necrosis factor-alpha (TNF-α). IL-6 is one of core mediators implicated in inflammatory and carcinogenic process in *H. pylori* associated gastric carcinogenesis via NF-κB, STAT3, and MAPK signaling pathways.[5] As seen in the Fig. 1A dealing with the changes of *IL-6* mRNA along with *H. pylori* infection in AGS cells, *H. pylori* infection significantly increased *IL-6* mRNA after 12 h and persisted up to 24 h. In this time, 24 h after 50 MOI *H. pylori* infection, increased *IL-6* mRNA was significantly decreased with increasing doses of WPE (Fig. 1B). Additionally, we measured the levels of secreted IL-6 in supernatant by ELISA and found *H. pylori* infection, 50 MOI for 24 h, significantly increased IL-6 levels ($p<0.01$), and IL-6 levels were significantly decreased with WPE in a dose dependent manner ($p<0.01$, Fig. 1C). In addition to IL-6, *H. pylori* infection is also associated with significant increases in TNF-α, IFN-γ, and IL-8, after which walnut extracts also led to significant decreases of these inflammatory mediators (Fig. 1D). Especially, gastric diseases after *H. pylori* infection are closely associated with COX-2 and c-*Myc* as possible oncogenes in inflammation based gastric carcinogenesis. As seen in Fig. 1E, *H. pylori* infection significantly increased COX-2 and c-*Myc* expression after 6 h of *H. pylori* infection, but WPE significantly decreased c-*Myc* expressions (Fig. 1E). Taken all together, WPE can significantly decrease *H. pylori*-associated inflammatory mediators.

STAT3 activations after *H. pylori* infection were significantly decreased with WPE. IL-6 led to STAT3 activation, which is closely intervened in inflammatory and carcinogenic events of *H. pylori* infection.[21,22] As seen in Fig. 2A, *H. pylori* infection is significantly associated with STAT3 activation, especially STAT3^{Tyr705}. In this condition, WPE significantly inactivated STAT3 tyrosine phosphorylation at tyrosine 705 site ($p<0.01$). In order to confirm the intervention of STAT3 activation via nuclear translocation, we repeated the measurement of STAT3 in nuclear fraction, as anticipated, WPE significantly inhibited

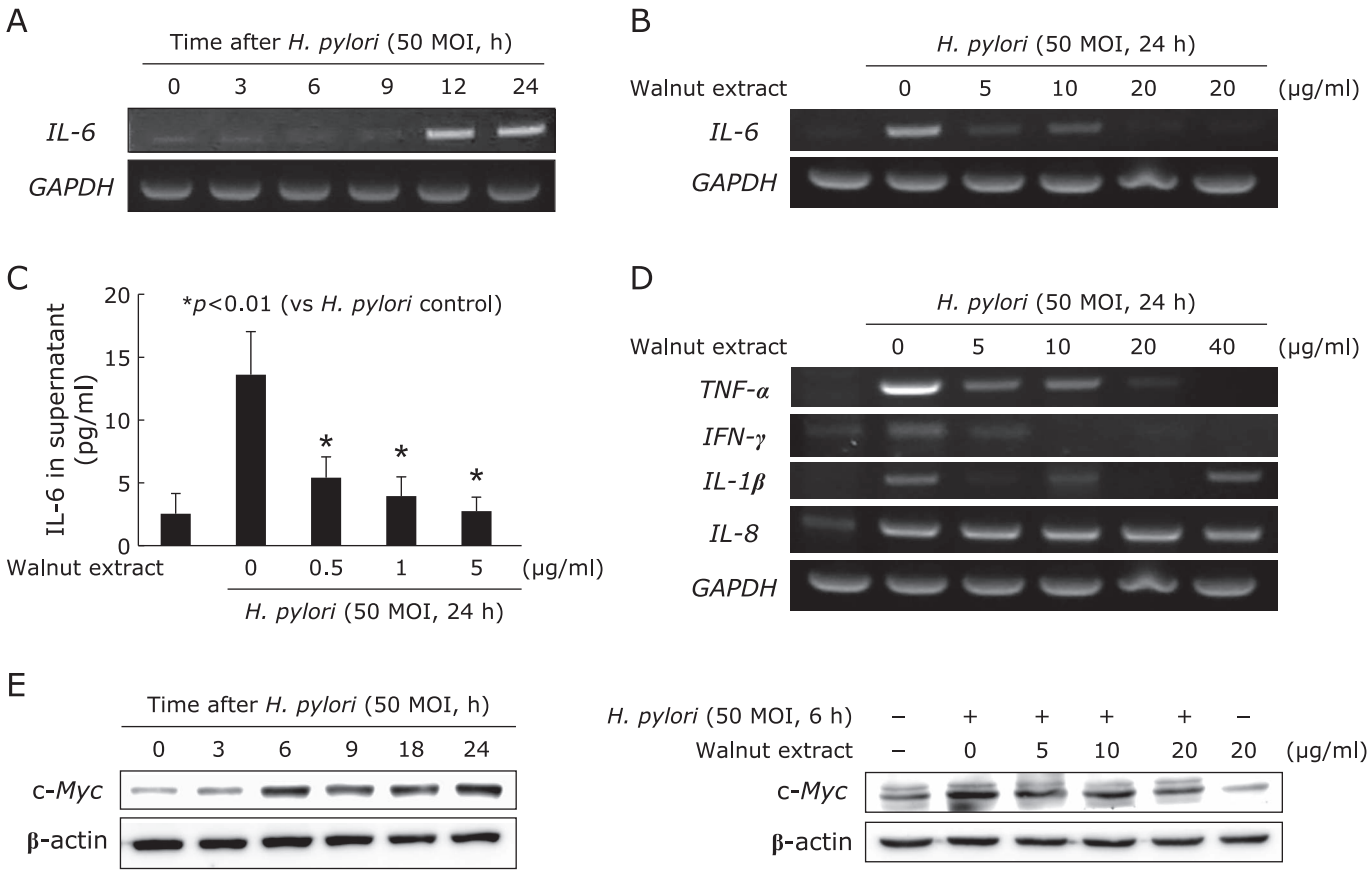

Fig. 1. Changes of IL-6 and other inflammatory mediators after WPE. (A) Changes of IL-6 mRNA after *H. pylori* infection in RGM-1 cells RT-PCR for IL-6 mRNA. (B) Changes of IL-6 mRNA according to WPE in the presence of *H. pylori* infection. (C) IL-6 levels in the supernatants of RGM-1 cells according to walnut dose in the presence of *H. pylori* infection. *p<0.01 vs *H. pylori* alone. (D) Changes of inflammatory mediators RT-PCR for *TNF-α*, *IFN-γ*, *IL-1β*, and *IL-8*. (E) Western blot for COX-2 and c-*Myc* Expression after times of *H. pylori* infection. Time dependent increases of expression noted after 6 h (left) and changes of c-*Myc* after walnut administration in the presence of *H. pylori* (right).

nuclear translocation of STAT3 (p<0.01, Fig. 2B). These findings were further confirmed by confocal imaging of STAT3 that walnut extract significantly inhibited nuclear translocation of STAT3 (Fig. 2C).

WPE induced SOCS1 contributed to *H. pylori*-STAT3 activation. SOCS1 has been identified as negative regulator of STAT3 and we have found WPE can induce SOCS1 as anti-inflammatory mechanisms.[23] Therefore, under the hypothesis that WPE can provoke anti-inflammatory action via SOCS1 induction and subsequent STAT3 inactivation, we measured the changes of *SOCS1* mRNA after *H. pylori* infection. As seen in Fig. 3A, host cells increased SOCS1 after 30 min *H. pylori* infection, but their levels were significantly decreased after 2 h. In this condition, WPE significantly preserved and induced SOCS1 in a dose dependent manner of WPE (p<0.01, Fig. 3A). These changes of *SOCS1* mRNA were further validated with the expressions of SOCS1 via western blot. As seen in Fig. 3B, 20 mg/ml WPE significantly increased SOCS1 expressions after 2 h of treatment (p<0.01), signifying WPE significantly induced SOCS1. In order to verify this SOCS1 induction with WPE contributed to p-STAT3 inactivation, we generated SOCS1 KO cells and compared the changes of p-STAT3^{Tyr705} and c-*Myc* between Mock- and siSOCS1-treated cells after *H. pylori* infection (Fig. 3C). As resulted, p-STAT3^{Tyr705} and c-*Myc* increases after *H. pylori* infection was significantly decreased in Mock-transfected cells, while no changes in p-STAT3^{Tyr705} and c-*Myc* were noted in siSOCS1 transfected cells.

WPE induced PPAR-γ led to significant inhibition of STAT3 activation via SOCS1 induction. In order to explain the contribution of SOCS1 to tackle *H. pylori*-associated STAT3 activation relevant to gastric inflammation, we checked the changes of peroxisome proliferator activated receptors-gamma (PPAR-γ), nuclear transcription factor of the steroid receptor superfamily, after WPE administration since we have preliminary data that WPE induced PPAR-γ. As seen in Fig. 4A, 30 min after WPE administration, PPAR-γ expressions were significantly increased (p<0.01). Since PPAR-γ is transcription factor existing in cytoplasm in order to transcript biological action, as seen in Fig. 4B, increasingly expressed in cytoplasm, but increasingly translocated in nucleus after WPE administration. In order to know the regulatory action of PPAR-γ activation in suppressing STAT3, we repeated the experiment to compare the STAT3^{Tyr705} phosphorylation between Mock-transfected and siPPAR-γ transfected cells according to WPE treatment. As result, WPE significantly decreased p-STAT3^{Tyr705} with WPE, but not in cells transfected with siPPAR-γ in spite of WPE administration (Fig. 4C) and these findings were further validated with the administration of BADGE, bisphenol A diglycidyl ether as PPAR-γ antagonist. As seen in Fig. 4D, activated STAT3^{Tyr705} after *H. pylori* infection was significantly inhibited with WPE administration, but not in cells treated with BADGE in spite of WPE co-administration, consistently suggesting that WPE is associated with PPAR-γ activation in inhibiting *H. pylori*-induced STAT3 activation.

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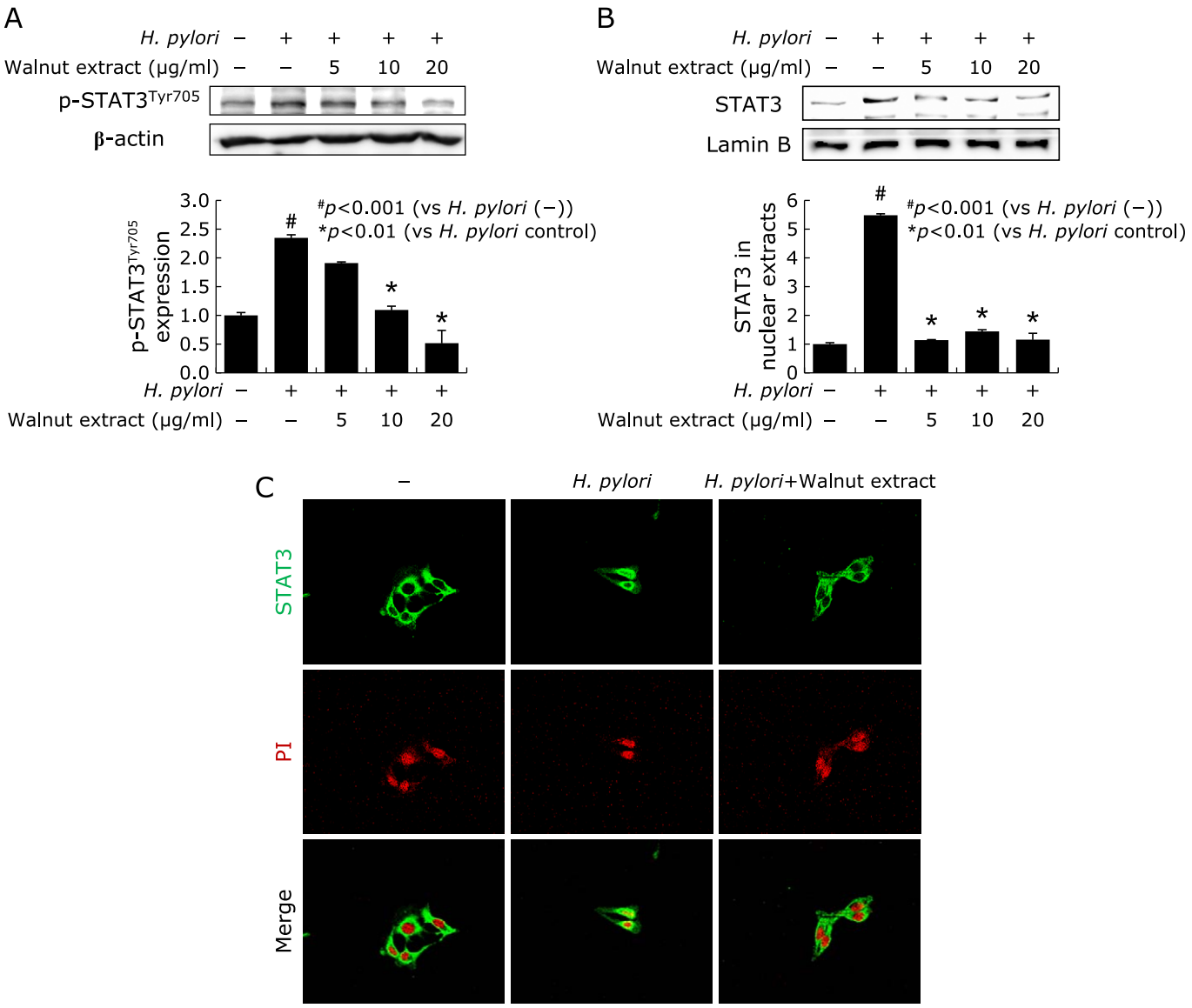

Fig. 2. Inhibition of STAT3 activations with WPE in the presence of *H. pylori* infection. (A) p-STAT^{Tyr705} was measured in cytoplasm extract after WPE in the presence of *H. pylori* Increased p-STAT3^{Tyr705} after *H. pylori* infection was significantly decreased with 10 or 20 μg/ml WPE. *p<0.01. (B) Nuclear expression of STAT3 STAT3 nuclear translocation was significantly decreased with WPE in nuclear fraction of RGM-1 cells infected with *H. pylori*. (C) Confocal imaging of STAT3 and PI according to WPE in the presence of *H. pylori* STAT3 expression localized mainly in the cytoplasm of non-stimulated RGM-1 cells, but their expressions moved to nucleus after *H. pylori* infection. However, even in the presence of *H. pylori*, STATs expression localized in the cytoplasm with WPE administration.

WPE regulated *H. pylori*-associated IL-6 and STAT3 activation via PPAR-γ activation and SOCS1 induction. Since previous data consistently showed PPAR-γ-transcribed SOCS1 is implicated anti-inflammatory action of WPE,[24] we compared the expression of SOCS1 between Mock transfected- and siPPAR-γ transfected cells in the expression of SOCS1 after WPE, as seen in Fig. 5A, SOCS1 mRNA was significantly decreased in siPPAR-γ transfected cells compared to Mock transfected cells. Next, we compared STAT3^{Tyr705} activation according to IL-6 and IL-6 KO condition. As noted in Fig. 5B, WPE significantly inhibited *H. pylori*-induced STAT3^{Tyr705} and c-*Myc* activation, but in cells transfected IL-6 or IL-6 receptor KO, no inhibition of STAT3^{Tyr705} was noted in spite of WPE administration and no induction of c-*Myc* induction.

Discussion

From the current investigation, we reached to summary as shown in Fig. 6 that concerted actions of PPAR-γ induction, SOCS1 induction, and subsequent inactivation of IL-6-associated STAT3 signaling with walnut extract administration in *H. pylori*-infected non-transformed gastric mucosal cells, RGM-1 cells can ameliorate either gastric inflammation or gastric tumorigenesis. Though proven in *in vitro* cellular system, our study provides hint for further consideration to meet clinical application that dietary intake of walnut can be a way to rescue stomach from *H. pylori* infection.[25] Gastric inflammation/mutagenesis mediated by COX-2, IL-1β, IL-6, IL-8, IFN-γ, and c-*Myc* become basis for gastric tumorigenesis, so called "inflammation-dysplasia-carcinogenesis via chronic atrophic gastritis" pathway,[26,27] in which chronic dietary intervention can be anticipating strategy for

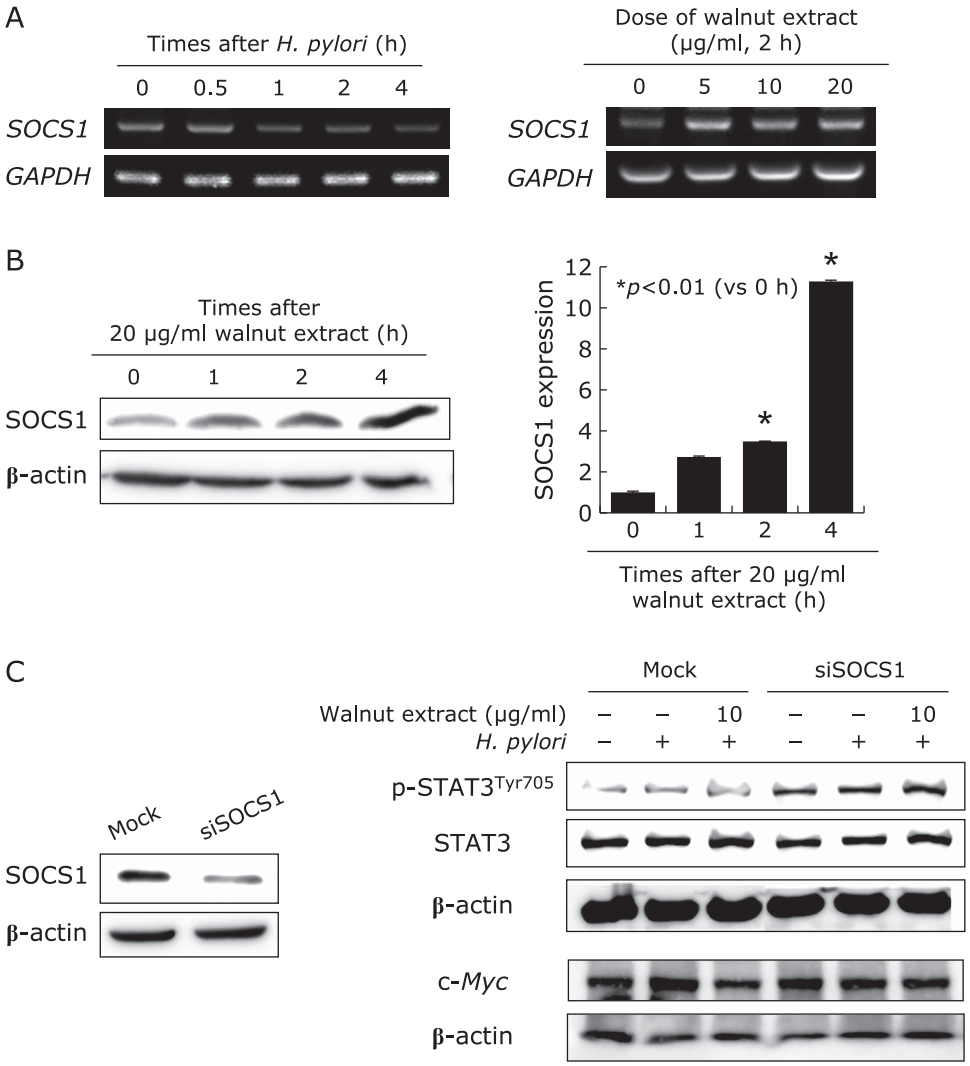

Fig. 3. Cancellation of SOCS after *H. pylori* and restoring of SOCS1 with WPE. (A) Changes of SOCS1 mRNA with *H. pylori* infection RT-PCR for SOCS1 (left) and changes of SOCS mRNA after WPE (right). (B) Western blot of SOCS1 after WPE Statistically significant increases of SOCS1 at 2 h of WPE. **p*<0.01. (C) STAT3 activation and the expression of c-*Myc* between Mock-transfected and siSOCS1 transfected RGM-1 WPE significantly decreased *H. pylori*-increased either p-STAT^{Tyr705} or c-*Myc* in Mock transfected RGM-1 cells, but these changes were abolished in siSOCS1-transfected cells.

prevention. Therefore, from our current study, we can conclude walnuts or its fresh phenolic extracts exerted significant rescuing action against *H. pylori*-associated gastric diseases including gastric cancer.

As molecular mechanisms of preventive strategies achieved with WPE, first, we have focused on IL-6/JAK/STAT3 pathway because they are responsible for transcribing inflammatory and mutagenic mediators such as COX-2, IFN-γ, iNOS, IL-1β, and c-*Myc* relevant to *H. pylori* infection. STAT3 is a transcription factor activated by various external stimuli including cytokines and growth factors. Upon activation, STAT3 is phosphorylated on Tyr705 or phosphorylated on Ser727 and translocates to nucleus where it regulates expression of target genes involved in cell proliferation, survival.[28,29] Although phosphorylation of STAT3^{Tyr705} has been essential for its dimerization, nuclear translocation, transcriptional activity and oncogenic function, phosphorylated STAT^{Ser727} stimulated tumor growth by modulating the activity of complex I and the intracellular accumulation of reactive oxygen species (ROS). JAK1/STAT3 is known to be an upstream signaling of NF-κB activation producing IL-8/IL-1β/IL-2 and generating ROS after *H. pylori* infection.[6,30,31] Conclusively, *H. pylori*-induced STAT3 activation is mediated through ROS-induced upregulation of IL-6 expression, necessitating inhibiting STAT3 to mitigate gastric damages after *H. pylori* infection. Consequent to IL-6 generation after *H. pylori* infection, augmented gp130-mediated cytokine signaling should be blocked in order to prevent *H. pylori*-associated inflammation and carcinogenesis.[9,32] In addition to these epithelial components, though not studied in the current investigation, STAT3 imparted a profound influence on immune response to *H. pylori* infection,[33] for instance, inhibition of dendritic cell maturation via IL-10 mediated STAT3 activation to facilitate *H. pylori* infection,[34] triggering the expression of the bactericidal lectin REG3γ to allow *H. pylori* to manipulate host immunity in order to favor bacterial survival in the gastric mucosal niche.[35,36] Especially among *H. pylori*, also used in our study, *H. pylori Cag*A activates STAT3 pathway in this propagation of gastric inflammation.[11] Since *H. pylori*-induced STAT3 activation is considered as significant signaling network in gastric cancer,[37–39] from the current study, WPE can contribute to significant inhibitory action of STAT3 via IL-6/gp130 inhibition.

PPAR-γ is a ligand-activated transcription factor. Since 15-deoxy-D12,14-prostaglandin J2 [15d-PGJ(2)] is a potent ligand for PPAR-γ, Cha B *et al.*[40,41] treated 15d-PGJ(2) in *H. pylori*-

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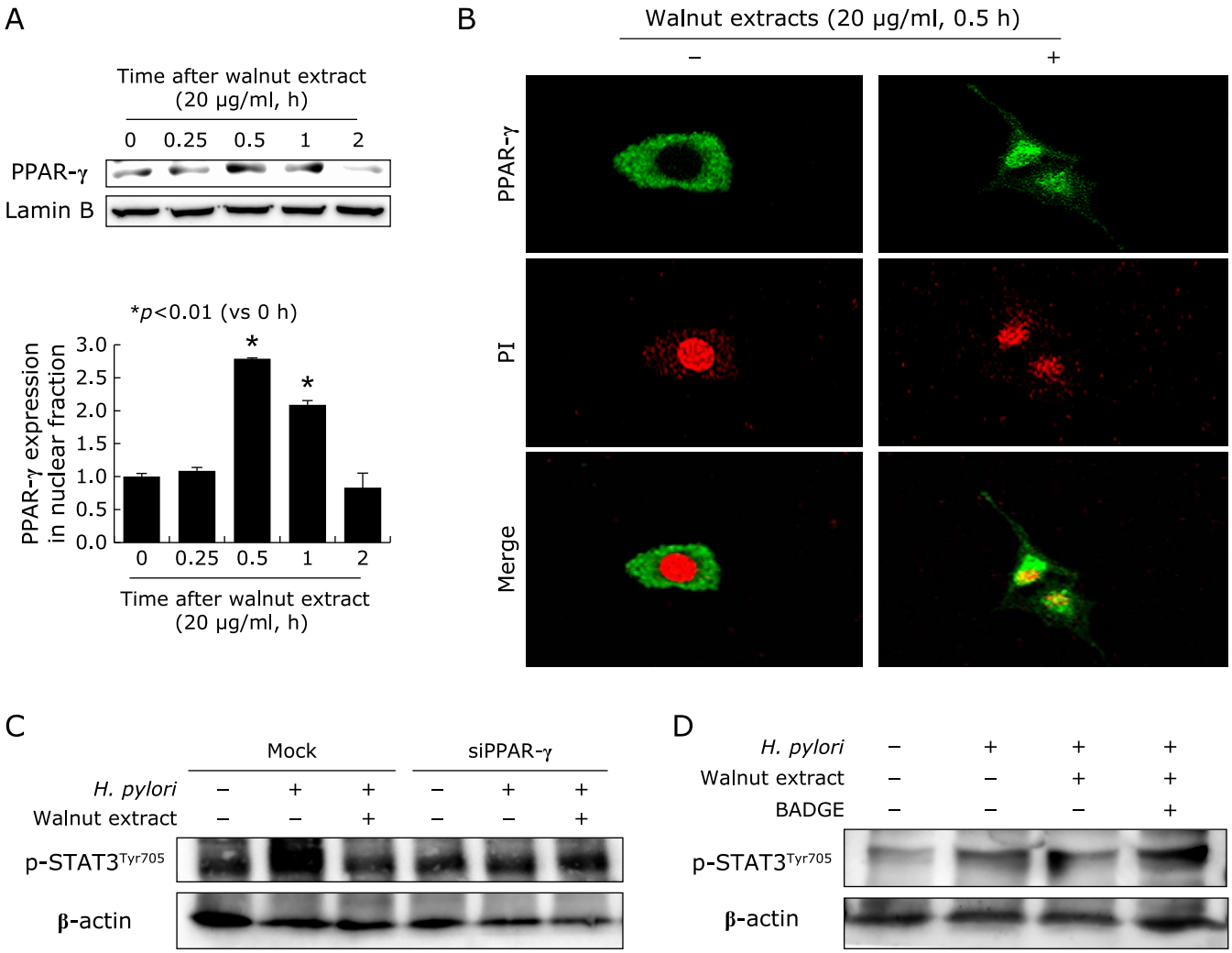

Fig. 4. Contribution of WPE-induced PPAR-γ on inactivation of STAT3. (A) Changes of PPAR-γ according WPE in RGM-1 cells. Significantly increased induction of PPAR-γ 30 min after WPE. *p<0.01. (B) Confocal imaging of PPAR-γ in RGM-1 cells. In normal RGM-1 cells, PPAR-γ localized mainly in the cytoplasm, its expression significantly increasingly increased in nucleus with WPE administration. (C) Changes of p-STAT3^{Tyr705}. In Mock transfected RGM-1 cells, *H. pylori* infection led to increased activation of STAT3, but decreased with WPE administration. On the other hand, in cells transfected with siPPAR-γ, no changes of STAT3 were noted, signifying STAT3 inhibitory action of walnut was mediated with PPAR-γ. (D) Changes of p-STAT3^{Tyr705} *H. pylori* infection clearly increased STAT3 activation at Tyr705 site and its activation was significantly decreased with WPE. However, BADGE as PPAR-γ antagonist also abolished STAT3 inactivation effect of WPE, signifying STAT3 inhibitory action of WPE was mediated through PPAR-γ activation. (E) Since previous study showed SOCS1 regulated STAT3, RGM-1 cells transfected with PPAR-γ significantly decreased SOCS1 expression.

infected gastric epithelial cells and PPAR-γ agonist significantly inhibited the activations of NADPH oxidase and RANTES expression via either inhibiting JAK1/STAT3 or NF-κB, concluding that 15d-PGJ(2) as PPAR-γ agonist can be beneficial for the treatment of *H. pylori*-induced gastric inflammation. Our preliminary and background study before the current investigation, we have published several results that *n*-3 PUFAs was quite beneficial in limiting *H. pylori* infection. Our group published rather clear conclusions that *n*-3 PUFAs exerted convincing evidence that *fat*-1 transgenic mice producing optimal levels of *n*-3 PUFAs in the stomach or intestine significantly rescued from either *H. pylori*-associated gastropathy or nonsteroidal anti-inflammatory drug-induced gastrointestinal damages.[18,42] Thinking clinical application from the above study, authors et al felt need to administer *n*-3 PUFAs as dietary intervention.[19] Ji *et al.*[43] studied the molecular mechanism underlying anti-inflammatory effects of *n*-3 PUFA, docosahexaenoic acid (DHA) in his study, against *H. pylori* infection and drawn similar result shown in our current study of *n*-3 PUFA containing WPE that DHA inhibited *H. pylori*-induced STAT3 phosphorylation in a PPAR-γ dependent manner.

In this investigation, with WPE administration, PPAR-γ transcribed SOCS significantly contributed to block STAT3 in *H. pylori* infection. Supported with our previous publication that anti-inflammatory signals of SOCS through STAT/JAK2 inactivation might be a key anti-inflammatory mechanism of probiotics, setting probiotics as a non-microbial strategy to *H. pylori* infection.[13] Generally, since SOCS1 not only participates in cell signaling, but also in ubiquitination mediated protein degradation process,[44–47] by which two extreme functions are possible, one is tumor growth, and the other is accelerated would healing.[48] Furthermore, focusing inflammatory regulation, anti-inflammatory as well as cytoprotection manifested as IFN-γ regulation and T cell differentiation prevails,[49,50] indicating a role in growth of gastrointestinal tissues, inflammatory bowel disease, gastritis, and cancer. The CIS (cytokine-inducible SH2 protein) and SOCS are a family of intracellular proteins, several of which have emerged as key physiological regulators of cytokine responses, including those that regulate the inflammatory

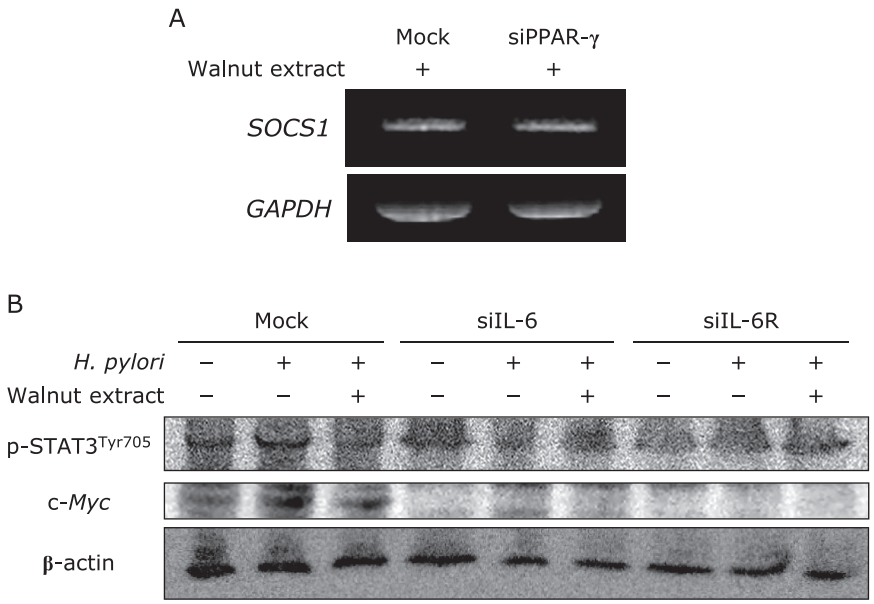

Fig. 5. WPE induced PPAR-γ to induce SOCS1 and simultaneously inactivated STAT3 via IL-6 inhibition. (A) WPE induced SOCS1 via PPAR-γ RGM-1 cells transfected with siPPAR-γ showed decreased expression of SOCS1 compared to Mock-transfected cells in the presence of WPE. (B) STAT3 and c-*Myc* expression according to IL-6 and IL-6 receptor. Cells transfected with either siIL-6 or siIL-6R showed no significant changes in STAT3 activation irrespective of WPE. Looking at c-*Myc* changes, WPE significantly decreased *H. pylori*-increased c-*Myc* in Mock-transfected cell, whereas no changes of c-*Myc* was noted in either siIL-6 or siIL-6 receptor.

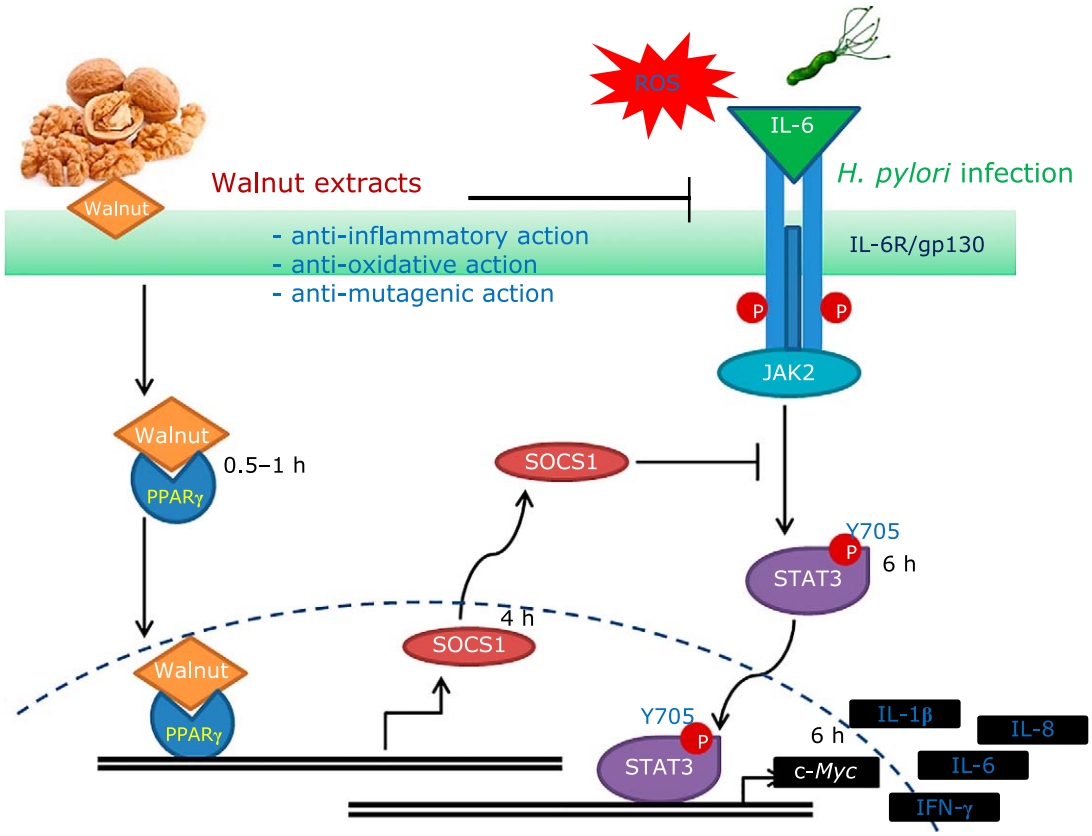

Fig. 6. Schematic presentation how WPE afforded significant protection from *H. pylori* infection. WPE possessing anti-inflammatory, antioxidative, and anti-mutagenic actions induced PPAR-γ immediately after administration, 30 min. PPAR-γ significantly transcribed SOCS1, which exerted significant inhibitory action against STAT3. *H. pylori* infection led to increased IL-6/IL-6R/gp130, resulting in increased phosphorylation of STAT3^{Tyr705}. These activations of STAT3 after *H. pylori* infection increased inflammatory mediators including COX-2, IL-1β, IL-6, and IFN-γ. Taken together, WPE contributed to inhibit inflammatory and mutagenic action of *H. pylori* via PPAR-γ activation, SOCS1 induction, and STAT3 inhibition.

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systems.[51,52] SOCS, consisting of eight members (SOCS-1 to SOCS-7 and CIS), all sharing a central SH2 domain and a C-terminal SOCS box, expression of SOCS is induced by various cytokines, and its overexpression studies in various cell lines have demonstrated their inhibitory roles on JAK/STAT.[53] Expression of SOCS-1 inhibited both IL-6-induced receptor phosphorylation and STAT activation, acting in a classic negative feedback loop to regulate cytokine signal transduction.[54]

In spite of the IARC (Lyon)'s definition that *H. pylori* is the definite carcinogen of gastric cancer, the simple eradication of the bug is not enough to prevent resultant gastric cancer, and increasing microbial resistance further limits the eradication application.[55] Therefore, walnut, probiotics, and phytoceuticals as non-pathogenic microbial feed, can affect the host in a beneficial manner. However, the mechanism of their anti-inflammatory actions is still unclear. In the current study, we hypothesized that SOCS signaling could be a feasible anti-inflammatory mechanism of walnut against *H. pylori* infection. PPAR-γ/SOCS1/STAT3 pathway was documented as potential candidate. In the literature, six native Iranian plants including *glycyrrhiza aspera*, *juglans regia*, *ligustrum vulgare*, *thymus kotschyanus*, *trachyspermum copticum*, and *xanthium brasilicum* were determined as anti-*H. pylori* plants,[56] among which *Juglans regia* L. is walnut. In study showing the *in vitro* anti-*H. pylori* activity of some selected medicinal plants on clinical isolates of *H. pylori*, the extracts of *Punica granatum* and *Juglans regia* had remarkable anti-*H. pylori* activity with mean of inhibition zone diameter of 39 and 16 mm at 100 μg/disc, respectively.[57]

Walnut (*Juglans regia* L.) contains a complex array of natural compounds and phytochemicals, for instance, in the metabolite-profiling analysis, walnut caused a significant increase in several polyunsaturated fatty acids (PUFAs), including DHA and 9-oxo-10(E),12(E)-octadecadienoic acid (9-oxoODA), as well as kynurenic acid, that exhibits a wide range of health benefits, including anti-inflammatory, antioxidative, and regenerating actions, by which dietary walnut supplementation (14% walnut) showed significant effects in recovery from dextran sulfate sodium-induced colitis,[58] significant protection against fenitrothion- or malathion-mediated immunotoxicity,[59,60] enriching intestinal microbiome for improving health condition,[61] reducing telomere length,[62] ameliorating colitis and colitis-associated cancer,[63] suppressing colon cancer cell growth,[64] and regulating anti-cancer stem cells.[65] Though not touched in the current investigation, cancer stem cell markers including CD133, CD44, DLK1, and Notch1 as well as the β-catenin/p-GSK3β signaling pathway were significantly down-regulated and the self-renewal capacity of CSCs was suppressed. Taken together with our investigation, WPE can impose significant cancer preventive action as well as anti-cancer effects in *H. pylori* infection.

Author Contributions

Study concept and design: JMP and KBH; acquisition of data: YMH, JMA, and JMP; analysis and statistical analysis: SJH; interpretation of data: YMH, JMP and YJS; drafting of manuscript: YMH and KBH. All authors approved the final version of this manuscript to be published.

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Abbreviations

CAG	chronic atrophic gastritis
COX	cyclooxygenase
H. pylori	*Helicobacter pylori*
JAK	Janus kinase
PPAR-γ	peroxisome proliferator-activated receptor gamma
SOCS	suppressor of cytokine secretion
STAT3	signal transducer and activator of transcription 3
TNF-α	tumor necrosis factor-alpha

Conflict of Interest

No potential conflicts of interest were disclosed.

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