C-Reactive Protein Knock Out Attenuate Temporomandibular Joint Inflammation in Rats

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Abstract

Background: C-reactive protein (CRP), as the biomarker for inflammation, high expresses in osteoarthritis (OA) related diseases with exact role been clouded. In this study, we evaluated the biological effect of CRP in temporomandibular joint osteoarthritis (TMJ-OA).

Methods: Freund's complete adjuvant (CFA) was used for TMJ inflammation induction in CRP knock out (CRP -/-) and control rats. And TMJ degenerative changes, such as the synovitis performance, TMJ disc morphological changes and cartilage degeneration were compared to elucidate the role of CRP played in TMJ-OA.

Results: Compared to control group, CFA induced TMJ inflammation caused systemic and local CRP expression increased. Based on this, CRP -/- rat performed less severe inflammation symptoms. Lower degree of pro-inflammatory cytokines (interleukins IL-1β and IL-6) expression and up-expression of anti-inflammatory cytokine IL-10 were detected in CRP -/- rat, which with less macrophage and activation and osteoclast differentiation.

Conclusion: These results indicated that control high elevated CRP level during inflammation should be benefit for TMJ-OA prevention and treatment.

Background

The temporomandibular joint (TMJ) is significant for our daily activities like eating, speaking and facial expression. Mandibular condyle, an interposed fibrocartilaginous disc, and glenoid fossa compose this exquisite joint [1]. As the fourth most common disease in stomatology, TMJ disorders (TMD) affects 5 to 12% of the population, and 65% of patients with rheumatoid arthritis (RA) have TMJ symptoms [2, 3]. Once TMJ diseases progresses to temporomandibular joint osteoarthritis (TMJ-OA), severe pain and dysfunction occurs, which caused heavy obstacles for daily life [4].

TMJ-OA is characterized as synovitis, TMJ disc morphological changes, cartilage degeneration and subchondral bone remodeling [5]. During TMJ-OA progression, synovial membrane is frequently the primary tissue invaded by inflammation, with the pathological changes including synovial hyperplasia, increased vascularity and inflammatory cells infiltration [6]. The TMJ disc, which undergoes morphological changes, becomes thickening, displacement, lengthening and perforation in severe stage [7]. While typical degenerative lesions of mandibular condylar cartilage include chondrocytes alignment irregularities, nested proliferation and hyalinization, accompanied by clefts and erosions occurred when progressed to severe stage [8]. Subchondral bone always has the resorption performance, with bone mineral density decreased and raised trabecular thickness, bone sclerosis and formation of osteophytes [9].

Inflammation is believed to be the vital factor for this pathological process, since pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukins IL-1β and IL-6 are elevated in synovial

fluid of TMD patients [6, 10]. While up-regulated inflammation factors aggravate TMJ-OA development, medical treatments targeted on inflammatory cytokines and signaling pathways show benefit effects on inflammation absorption and TMJ-OA recovery [11, 12]. TNF-α inhibitors and IL-1 receptor antagonist are the well-established therapeutic approaches for RA treatment [13–15], and antibodies to TNF-α, IL-1β and IL-6 show positive effects during RA therapy investigation [16, 17]. Thus, inflammatory control, especially the treatments focus on inflammatory factors reduction, acts as an important part for TMJ-OA therapy.

C-reactive protein (CRP), a reactive protein in the acute phase of inflammation, could be induced by inflammatory cytokines (TNF-α, IL-1β and IL-6), synthesized by hepatocytes and released into the circulatory system [18]. Although it has long been regarded as a marker of acute inflammation, recent studies have shown that CRP also plays an important regulatory role in a variety of chronic inflammatory related diseases [19, 20]. It has long been recognized that elevated serum CRP correlates positively with RA severity and progression, and systemic CRP level is significantly increased in OA patients [21, 22]. Several clinical studies involving TMJ have also confirmed that raised level of serum CRP is associated with TMJ pain and bone loss in patients [23, 24].

Despite all these “association”, the exact biology role of CRP on OA seems in the cloud. No clinical research directly reveals the effect of CRP on OA, and animal studies gave mixed results so far [25]. Inhibition CRP expression decreased bone erosion in rat OA and promote osteoclastogenesis in vitro, demonstrated the detrimental role of CRP on OA [25, 26]. While the study which shows the collagen-induced arthritis (CIA) was exacerbated in CRP deficient mice and transgenic expression of human CRP rescued OA development, suggested the protective effect of CRP [27, 28]. The exact role of CRP on TMJ-OA has not been retrieved at the moment. In this study, CRP knockout rats were constructed and cultivated by TALENs (transcription activator-like effector nucleases) technique. Freund’s complete adjuvant (CFA) was used for TMJ inflammation induction in CRP deficient and control rats. And TMJ degenerative changes were compared to elucidate the exact role of CRP played in TMJ-OA.

Methods

Experiments were conducted with the ethical protocol approved by the Animal Ethics Committee at Chongqing Medical University (AECCMU-2020-004). All the methods were in accordance with the approved guidelines.

Animals and TMJ inflammation induction

Adult female Sprague Dawley rats aged 10-12weekes and weighing 350-400g were used as control to CRP knock out (CRP-/-) rats of the same age and weight. The CRP-/- rats were offered by professor Zhigang Yang from Chongqing medical school. DNA sequencing results of these rats showed that with several basic groups deficiency in exons, CRP protein expression decreased in hepatic tissue together with decline in serum CPR level (Supplemental Fig.1). Animals were housed in Experimental Animal Center of Stomatological Hospital of Chongqing Medical University, maintained in a barrier room at 25 °C with 40% humidity of 12-hour light/dark cycle, with free access to food and water available ad libitum.

Inflammation was induced by 50 μl CFA (Sigma, MO, USA) injection into the upper compartment of bilateral TMJs [29]. And rats without inflammation induction were received saline injection of the same volume. Thirty-two rats were included and divided into 4 groups. Control group was the SD rats with saline injection, and CFA group with CFA injection. CRP-/- rats injected with saline were classified as CRP group, while the ones with CFA injection were into CRP+CFA group.

Tissue harvest and disc weight measurement

The heads were hemisected after sacrificed by carbon dioxide inhalation 7 days after inflammation induction. And one side of TMJ capsules opened, exposed naked condyle and separated disc tissue. The contralateral heads were not disarticulated and fixed *in situ* in 4% paraformaldehyde (PFA, Affymetrix, USA) at 4°C for slides section. Separated disc were clean up carefully and weighed with excess water removed. Separated disc and synovia from the same rat were then gathered together for PCR analysis.

Paraffin section preparation and staining

The PFA-fixed hemi-heads were decalcified with 14% EDTA (pH 7.5) for up to 2 months. After gradient-decalcification, paraffin was used for embedding samples with sagittal section. Five μm thick sections were obtained before stained with hematoxylin and eosin (H&E), safranin O and fast green (Solarbio, China) to observe histological change in cartilage using standard methods. And tartrate resistant phosphatase (TRAP) staining (Solarbio, China) was performed for osteoclasts activity estimation. Sections from equivalent regions of the TMJ were compared between animals.

Histological analysis

Slides with H&E staining were used for histological analysis by 2 blinded examiners separately. Disc thickness measurement was done in anterior, intermediate and posterior band in 6 randomly selected sections every joint (N=6/group) referred to Wang's study [7]. And the extended lines of intermediate zone in cartilage were gauged for the thickness of total cartilage, fibrocartilage layers and hypertrophic chondrocytes layers with safranin O and fast green staining slides.

For inflammation score of synovial membrane, scores were semiquantitatively evaluated with following parameters: one scale for inflammatory infiltration: no infiltration = 0; discrete infiltration =1; moderate infiltration = 2; intense infiltration = 3. Another scale for synovial membrane thickness: no thickening = 0; discrete thickening =1; moderate thickening = 2; intense thickening = 3 [8]. And the total number of mononucleated cells in synovial membrane of 100*100 μm square was counted for infiltrated inflammation cells measurement (N=6/group, every joint with 6 randomly selected sections).

Immunohistochemistry

Immunohistochemical staining was performed with sections been de-paraffinized, Rehydrated. Slides were incubated with 0.3% hydrogen peroxide for 20 minutes (min), before processing with serum to reduce unspecific binding. The slides were then incubated overnight with anti-CRP 1:200 (abcam, USA),

anti-IL-1β 1:100 (Bioss, China), anti-IL-10 1:150 (Bioss, China), anti-TNF-α 1:100 (Bioss, China), anti-CD68 1:100 (abcam, USA), anti-Inducible Nitric Oxide Synthase (iNOS) 1:100 (Affinity, USA), anti-receptor activator of NF-κB (RANKL) 1:100 (Bioss, China). Then slides were washed and incubated with goat anti-mouse second antibody for 30 min (Zhongshan Biotechnology, China), visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate (Zhongshan Biotechnology, China) and counterstained with hematoxylin.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from disc and synovia with Trizol reagent (Invitrogen, USA) following the instructions. RNA qualification, reverse transcription and polymerase chain reaction were performed as previously described in detail [30]. The sequences of the commercially synthesized primers are listed in Supplemental Table.

Enzyme linked immunosorbent assay (ELISA)

After centrifuging the blood of rats, the serum was acquired and diluted 4000 times with double distilled water. Diluted sample was added into each well of enzyme label plate, incubated at 25 ℃ for 30 min before washed for 5 times. Then 100 μl of substrate was added into the wells, and the color was developed in dark at 25 ℃ for 10 min. After that 100 μl of termination solution was applied in each well. The optical density at 405 nm was measured with 650 nm wavelength correction.

Western-Blot analysis

Protein samples from hepatic tissue were extracted using RIPA lysis buffer (Thermo Fisher Scientific, USA). The total protein was separated and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% BSA in PBS for 60 min at room temperature and then were probed with anti-CRP (1:1000; Abcam, USA) or anti-β-actin (1:5000; Zhengneng, China) at 4°C overnight. Then followed by the addition of goat anti-rabbit IgG H&L secondary antibodies (1:500; Abcam, USA) for 60 min, immunoreactive proteins were detected with a chemiluminescence kit (Millipore, USA) by enhanced chemiluminescence (Amersham Pharmacia Biotech, USA) reaction.

Statistical analysis

SPSS 23.0 software (SPSS Inc., USA) was used for data analysis. Data were expressed as mean + standard deviation (SD). The normality of data distribution was tested by Shapiroee-Wilk test, and Levene's test was used to assess homogeneity of variance. Statistical comparisons were performed using independent t-test (two groups comparison) or One-way ANOVA analysis (beyond two groups comparison) followed by multiple comparisons using the Tukey's test.

Results

Inflammatory manifestations in TMJs

According to the study on time-dependent degeneration of TMJ induced by CFA in rat [8], obvious symptoms of inflammation changes appeared 7 days after injection, and swelling gradually subsided following that time [7], we chose to collect samples 7 days after CFA induction. The CRP expression in hepatic tissue and serum CRP level has the distinct elevation in CFA group (Supplemental Fig.2). Marked increase could be found with linear head width between bilateral TMJs in CFA and CRP+CFA group, which demonstrated the swelling of TMJ areas (Fig.1 A-D). When the articular cavity was exposed, swollen and hyperplastic synovia membrane (Fig.1 E-H) and thickening opacity disc (Fig.1 I-L) could be found in CFA and CRP+CFA group. Compared to CFA group, the related inflammation changes in CRP+CFA seems milder with lighter disc weight (Fig.1 M).

Tissue, cellular and molecular change in inflamed TMJ

The H&E slides involved disc and cartilage shows thickening and deformation disc in CFA group, and abnormal stratification of cartilage revealed, with cartilage surface seems adhered to some aera of articular disc tissue. While in CRP+CFA group, only thicken disc could be found compared to CRP group and Control group (Fig2. A-D). With safranin O and fast green staining hypertrophic chondrocytes layers decrease in CFA group has been observed, while CRP+CFA group shows no obvious change (Fig2. E-H).

The whole disc was thickening based on the measurement of anterior, intermediate and posterior band after CFA induction without distinct difference when CRP deleted (Fig2. I & K). Total cartilage thickness decline in CFA group was found in CFA group, which contributed by decreased of hypertrophic chondrocytes layers, while the fibrocartilage layers had the significant increase in thickness compared to Control group. The differences between CRP and CFA+CRP group was not that obvious, but increased fibrocartilage layers could be found in CRP group (Fig2. J & L).

Compared with disc and cartilage, synovial membrane was the most sensitive tissue for inflammation induction. Partly because of the CFA were injected to the superior articular cavity, the synovial membrane in upper compartment experienced severer inflammation (Fig3. A-H). Several characteristic change of inflammation could be easily figured out in CFA and CFA+CRP group, which included apparent infiltration of mononucleated cells, marked proliferation of lining cells and abundant lipid droplets in synovium, with CFA group offered more apparently changes (Fig3).

Inflammatory cytokines expression in disc and synovial membranes

CRP mRNA level increased after CFA induction (Fig4. A). And the inflammatory cytokines IL-1β and IL-6 raised to higher level in CFA group than CFA+CRP group. And IL-10, which is considered as anti-inflammation factor, got a distinct decrease in CFA group, and when CRP knocked out its expression increased (Fig4. C, F & F). Elevated expression of TNF-α and IL-2 was found in CFA and CFA+CRP group without significant difference (Fig4. B & D). The IHC results of CRP, TNF-α, IL-1β and IL-2 showed similar protein expression trend as mRNA level (Fig4 G-V).

Macrophage and osteoclast activity in inflamed TMJ

CD68 and iNOS are the activation marker protein of M1-like macrophages. More CD68 positive cells were found in CFA group than CRP+CFA group. While iNOS expression was comparably obvious in CFA group only (Fig.5 A-H).

RANKL, which induced osteoclast activation and cartilage degeneration in OA, highly expressed in CFA group rather than CRP+CFA group (Fig.5 I-L). With TRAP staining, more positive cells, which represents osteoclasts, appeared in CFA group compared to CRP+CFA group (Fig.5 M-Q).

Discussion

The contradictory results of researches clouded the association between CRP expression and OA progression for a long time. Nicholas's study with CRP deficient mice demonstrated that CRP exerts beneficial effect in CIA, which dampened inflammation responses [28]. Another study showed the protective effect of CRP exerted during the beginning of arthritis using rabbit CRP-transgenic mice [27]. While the anti-CRP antibodies injection attenuated bone erosion and bone resorption and prevent bone loss in rats. And with liver-targeted CRP siRNA, which decreased CRP expression in hepatocytes, the similar effect as anti-CRP antibodies was founded in Liang's study [25]. Also, CRP signaling was detected highly active in synoviocytes from RA patients, and exogenous addition of CRP could induce synovial inflammation via activation of NF-κB signaling [32].

Some researchers attributed this phenomenon to the different stages of CRP intervened, and suggested CRP may play protective role during early stage of RA, but confer detrimental effect during active RA [25, 28]. Related studies, which revealed different conformations of CRP bind to corresponding receptors and activate distinct signaling, may partially elucidated both proinflammatory and an-inflammatory effects of CRP [32, 33]. Another factor that cannot be ignored is genetic variation. Base on Rhodes's study, 232% difference of CRP level in RA patients attributable to genetics alone [34]. Thus, serum CRP level should be influenced by underlying inflammation together with genetic variation [18]. The large variations of serum CRP level in SD rats and DBA/1 mice was detected in Liang's study, and CRP interference therapy attenuated bone damage in high CRP level animals with CIA [25]. Besides, metabolism influence should be taken into consideration, since human CRP aggravated OA development in high-fat diet mice [35]. These researches indicated that CRP level elevation during inflammation should be the premise for exploration CRP effect on OA, which influenced by inflammation progression, genetic variation and metabolism.

In the present study, we demonstrated that CFA induced TMJ inflammation caused systemic and local CRP expression increased. Based on this, CRP -/- rat performed less severe inflammation symptoms and lower degree of inflammatory cytokines expression. Even though with milder inflammatory manifestations, hyperplastic synovia membrane, thickening disc, diffuse infiltration of inflammatory cells and abundant lipid droplets appeared in CRP + CFA group, with inappreciable change on cartilage (Fig. 1–3). These results indicated that control high elevated CRP level during inflammation should be benefit at least for prevention TMJ-OA rapid progressed to hard tissue.

Related studies demonstrated that interaction of CRP with its receptor promoted proinflammatory cytokines (IL-1β, IL-6 and TNF-α) production, leading to amplification of inflammatory reaction [26, 36]. And in CFA induced TMJ inflammation, IL-1β and TNF-α acted as the specific up-regulated proinflammatory factors [11, 12, 37]. By PCR analysis and IHC staining in synovial membrane, we found lower level of IL-1β and IL-6 in CFA + CRP group compared to CFA group (Fig. 4). But TNF-α did not performed obvious decline, which demonstrated that other factors beyond CRP, regulated TNF-α in this context. And IL-10, which acts as anti-inflammation factor and declined in CFA induced inflamed TMJ [11], increased when CRP deleted (Fig. 4). The combination effects of these inflammatory factors reduces symptoms in CFA + CRP group.

M1-like macrophages, which act as the main producers of inflammatory mediator, such as IL-1β, IL-6 and TNF-α, play a major role in joint inflammation [37, 38]. In contrast, M2 macrophages, which produced IL-10, adapt to an anti-inflammatory role [39]. We found CD68 and iNOS highly expressed in CFA group which showing M1 macrophage activation. And the CD68 and iNOS expression level in CRP + CFA group comparably reduced (Fig. 5A-H), which accounted for lower inflammatory reaction.

RANKL, as the core component of NF-κB signaling, mediates the synthesis of catabolic factors and osteoclasts activation, resulting cartilage degeneration in OA [40]. Only the cartilage of CFA group showed some degenerative change with more RANKL positive cells found in cartilage (Fig. 5I-L). And TRAP positive cells, which represents osteoclasts, increased in CFA group compared to CRP + CFA group (Fig. 5M-Q). These results demonstrated that increased CRP level promoted RANKL expression and contributed to cartilage degeneration. While the study on the role CRP played in osteoclastogenesis revealed that CRP neutralized RANKL to inhibit RANKL-induced osteoclastic differentiation, and monomeric CRP (mCRP) promoted this process in the absence of RANKL [32]. In the situation that concentration of mCRP higher than that of RANKL, CRP inhibition exerted a protective effect in OA [25]. Similar effect of CRP functions as a negative regulator of macrophage activation was found when it binding to apoptotic DNA [38]. Thus, besides the factors (inflammation progression, genetic variation and metabolism) mentioned previously, the exact role of CRP played on OA is also conformation-dependent and binding factors influenced.

Conclusions

With the results of the present study, we concluded that CRP control is benefic in the CRP high elevation TMJ-OA inflammation for milder inflammatory reaction and less cartilage degeneration. And other strategies focused on inflammatory factors and immune response should be combined for better treatment outcome.

Abbreviations

CRP: C-reactive protein; OA: osteoarthritis; TMJ-OA: temporomandibular joint osteoarthritis; CFA: Freund's complete adjuvant; IL: interleukins; TMJ: temporomandibular joint; TMD: TMJ disorders; RA: rheumatoid

arthritis; TNF-α: tumor necrosis factor alpha; CIA: collagen-induced arthritis; TALENs: transcription activator-like effector nucleases; H&E: hematoxylin and eosin; TRAP: tartrate resistant phosphatase; iNOS: Inducible Nitric Oxide Synthase; RANKL: receptor activator of NF-κB; DAB: diaminobenzidine tetrahydrochloride; RT-qPCR: Real-time quantitative polymerase chain reaction; ELISA: Enzyme linked immunosorbent assay; SD: standard deviation; ANOVA: One-way analysis of variation; mCRP: monomeric CRP

Declarations

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Contributions

YH, MZ and ZJ: Contributed to conception, design, data acquisition and interpretation, performed all statistical analyses, drafted and critically revised the manuscript; LF and LH: Contributed to conception, design, data acquisition and critically revised the manuscript; JS: Contributed to conception, data acquisition, and critically revised the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

According to the recommendations of the Chinese Academy of Sciences, the protocol of this study was authorized by the Animal Ethics Committee at Chongqing Medical University (AECCMU-2020-004). Informed consent was signed by all participants.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Figures

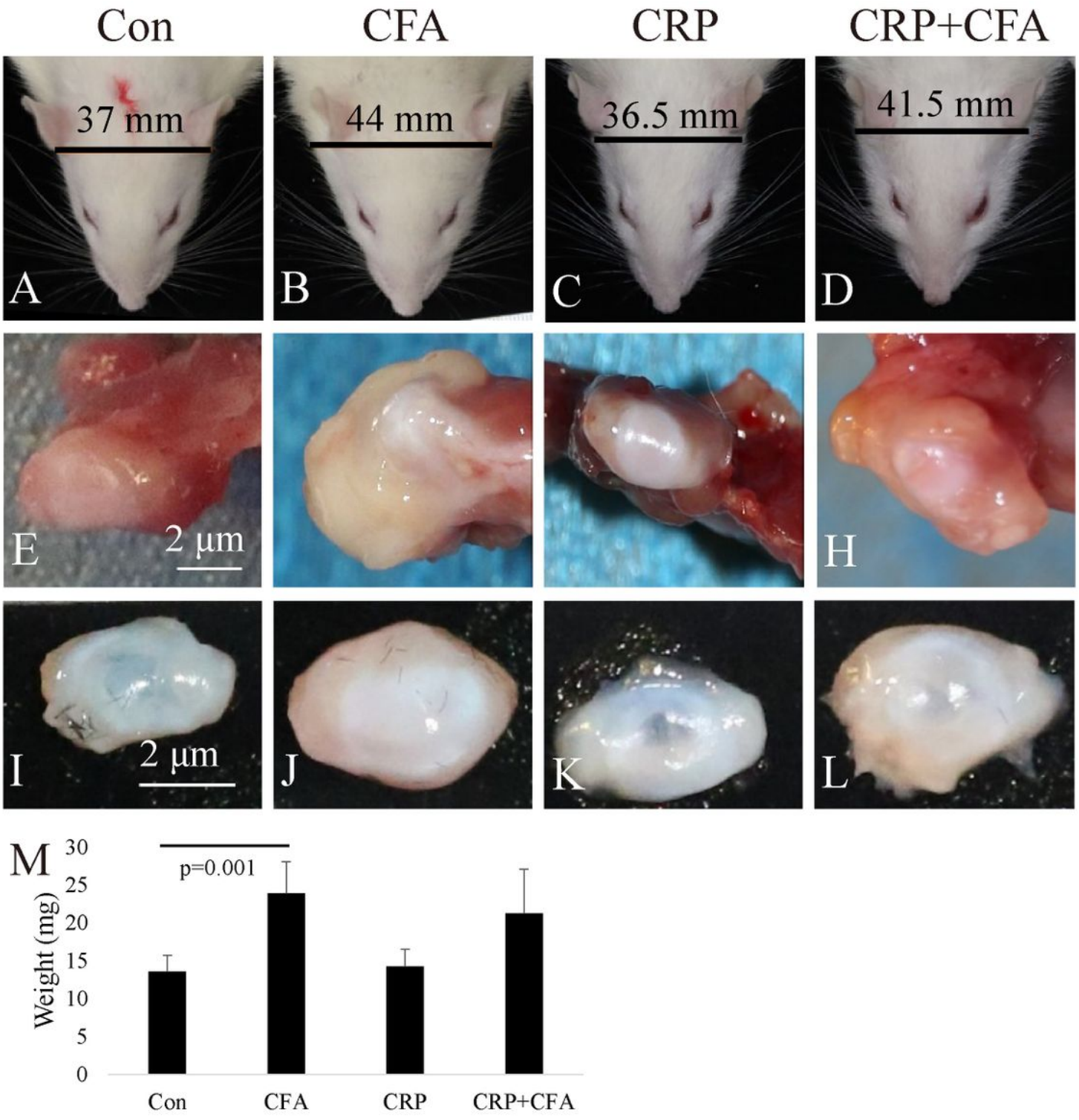
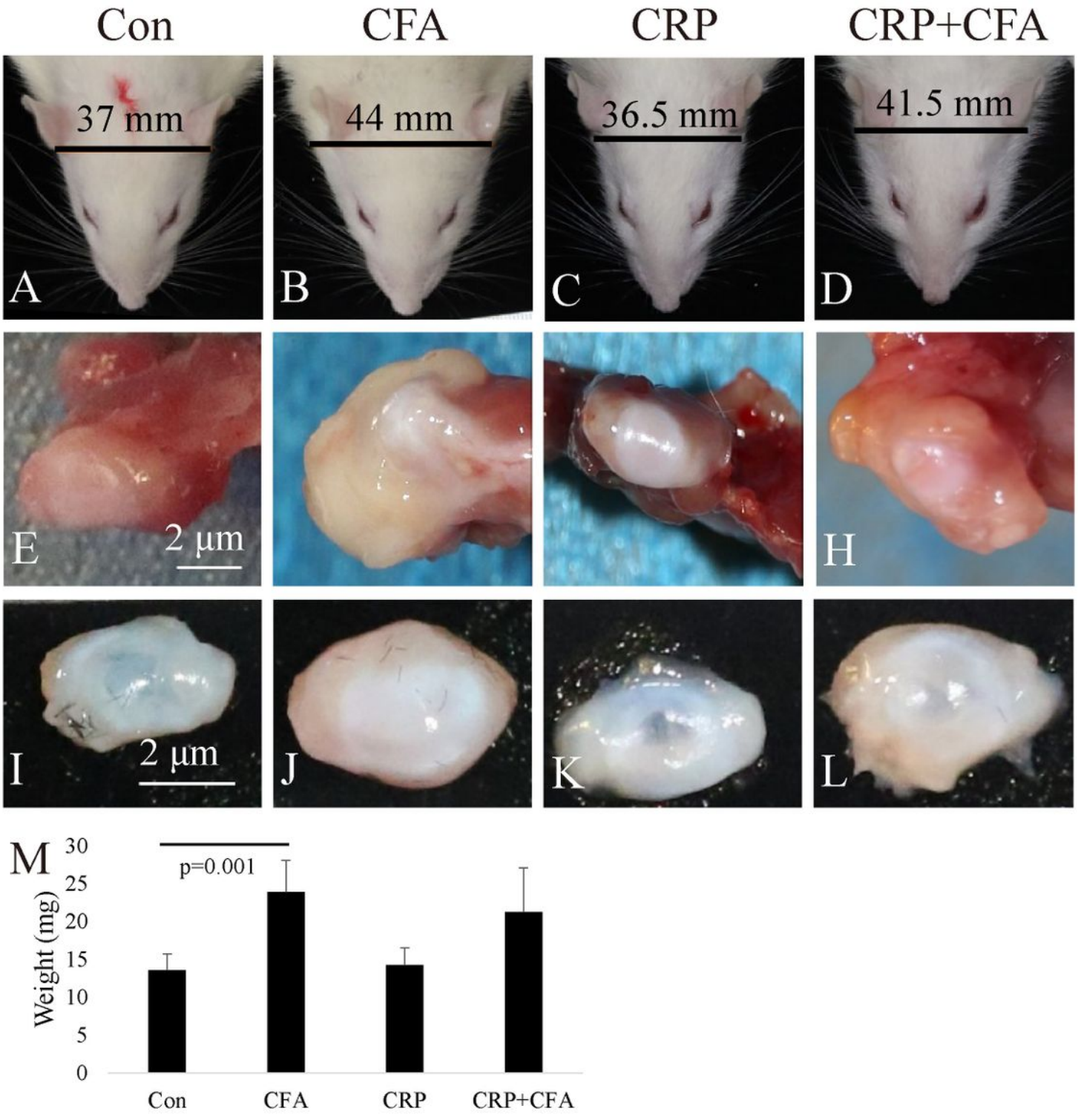


Figure 1

Inflammatory manifestations in rat TMJs. (A-D) Representative photos of rats' heads with linear head width marked the distance between bilateral TMJs. (E-H) Representative photos of exposed TMJs, note swollen and hyperplastic synovia membrane in CFA and CFA+CRP group. (Fig.1 I-L) Representative photos of discs from different groups, thickening opacity disc from CFA group was obvious. (M) Net weights of disc showing significant higher in inflammation groups than controls (N=8; mean+SD).



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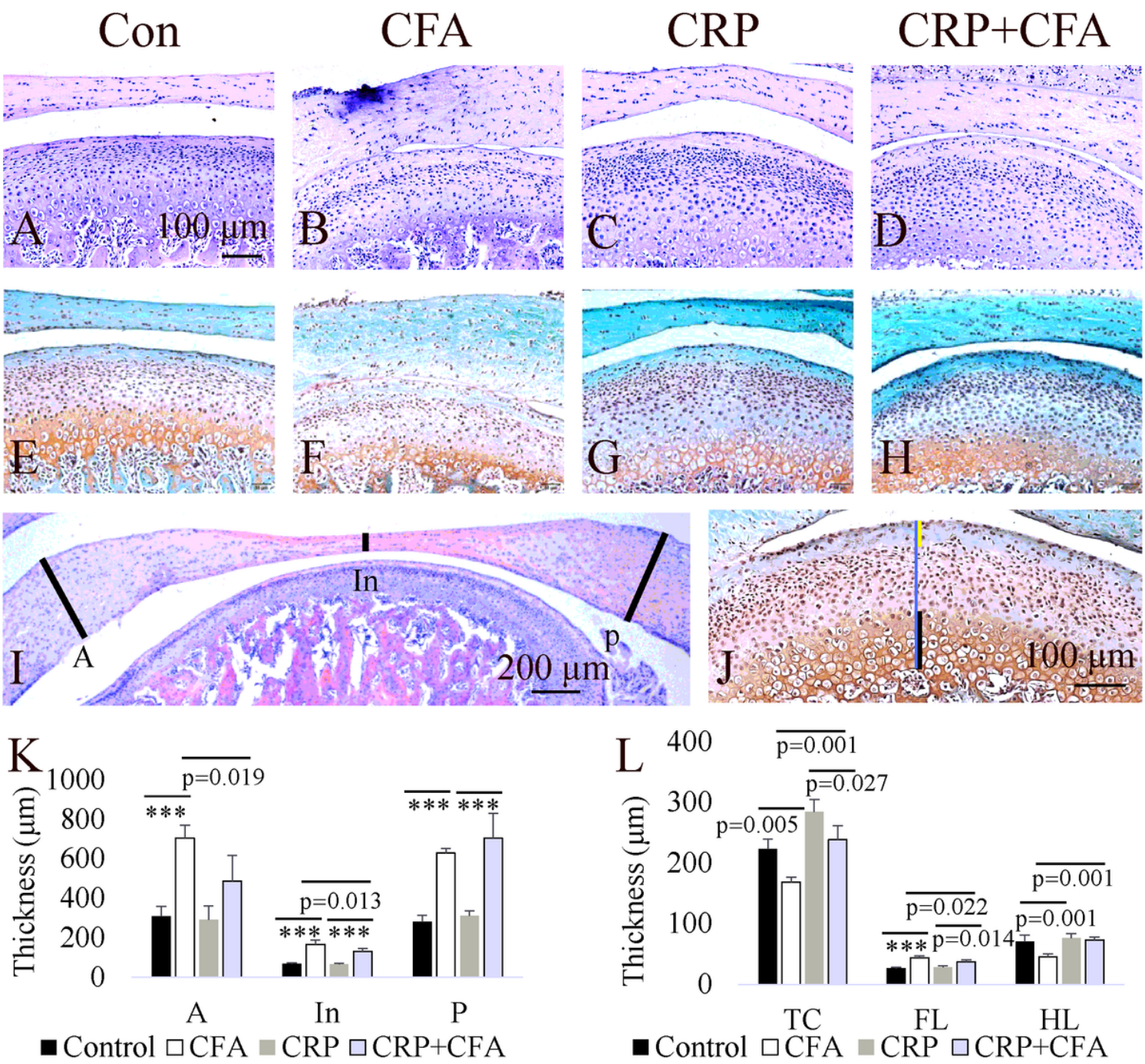
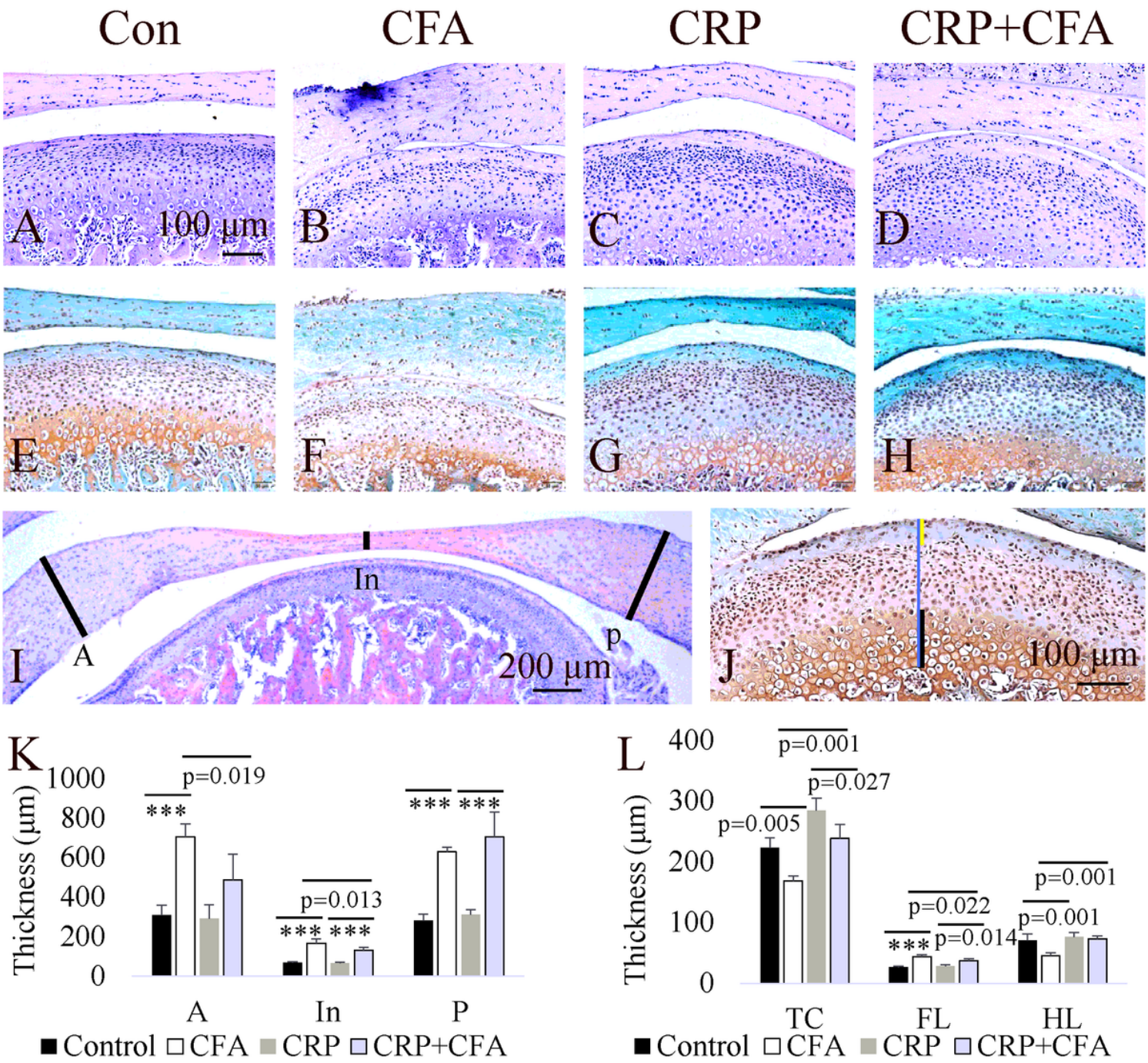


Figure 2

Histological changes of TMJ disc and cartilage (A-D) Representative images of H&E staining slides shows middle part of TMJ disc and cartilage. (E-H) Representative images of safranin O and fast green staining slides involved TMJ disc and cartilage. (I) Photomicrograph showing the way for disc thickness measurement (A: anterior band; In: intermediate zone; P: posterior band). (L) Photomicrograph of cartilage thickness measurement, with blue line indicated total cartilage thickness (TL), yellow line for fibrocartilage layers thickness (FL) and black line for hypertrophic chondrocytes layers thickness (HL). (K) Statistical results of disc thickness in every group. (L) Statistical results of cartilage thickness in every group. (N=6; mean+SD; ***p < 0.001).



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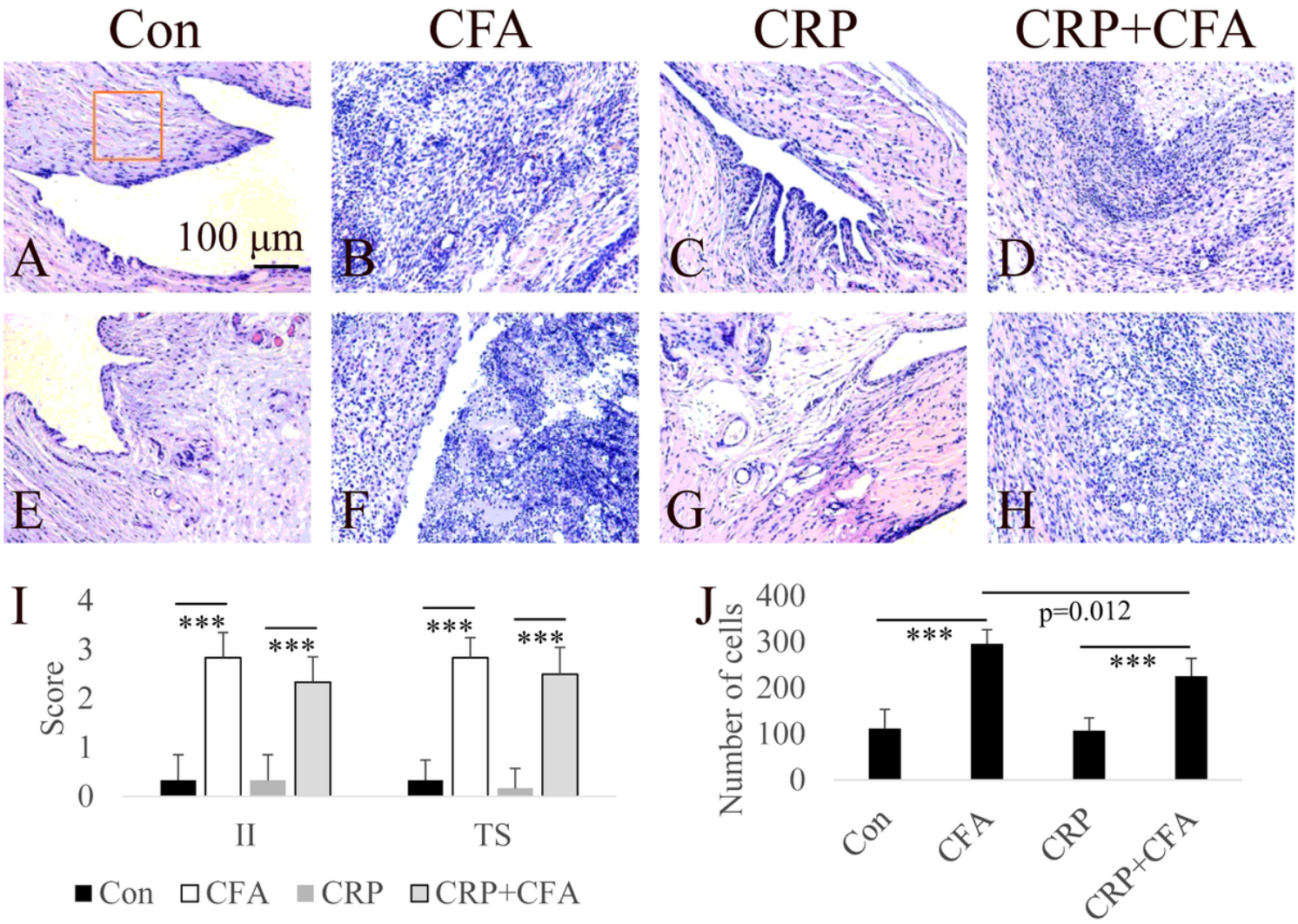
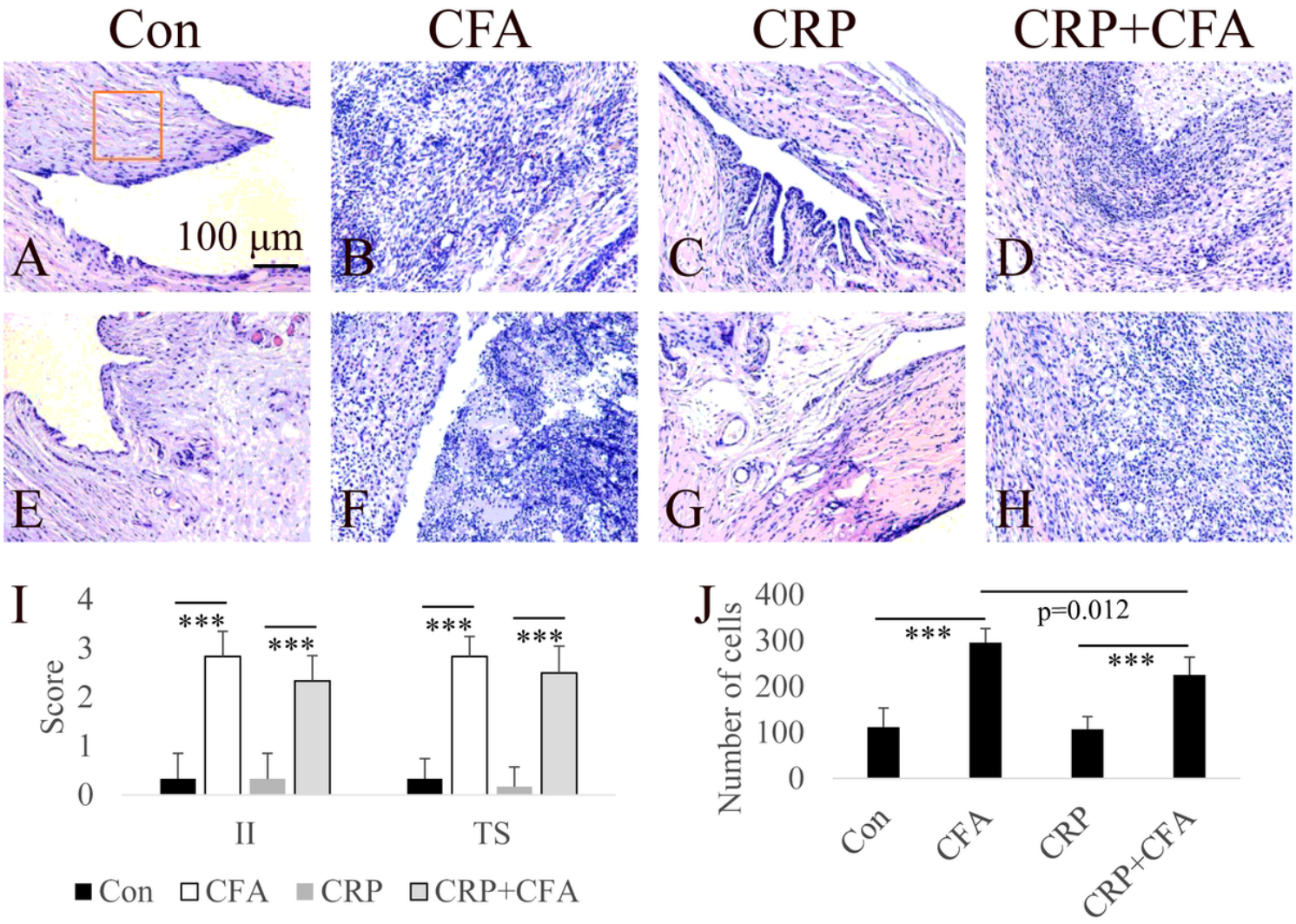


Figure 3

Histological changes of synovial membrane (A-D) Representative images of H&E staining slides showing synovial membrane from anterior superior region. The orange box indicated 100*100 μm square for infiltrated inflammation cells counting. (E-H) Representative images of synovial membrane from posterior superior region. (I-L) Representative images of synovial membrane from anterior inferior region. (M-P) Representative images of synovial membrane from posterior inferior region. (Q) Inflammation score based on inflammation infiltrate (II) and thickening of synovial membrane (TS). (R) Number of mononucleated cells in synovial membrane of 100*100 μm square measured as inflammatory infiltration. (N=6; mean+SD; ***p < 0.001).



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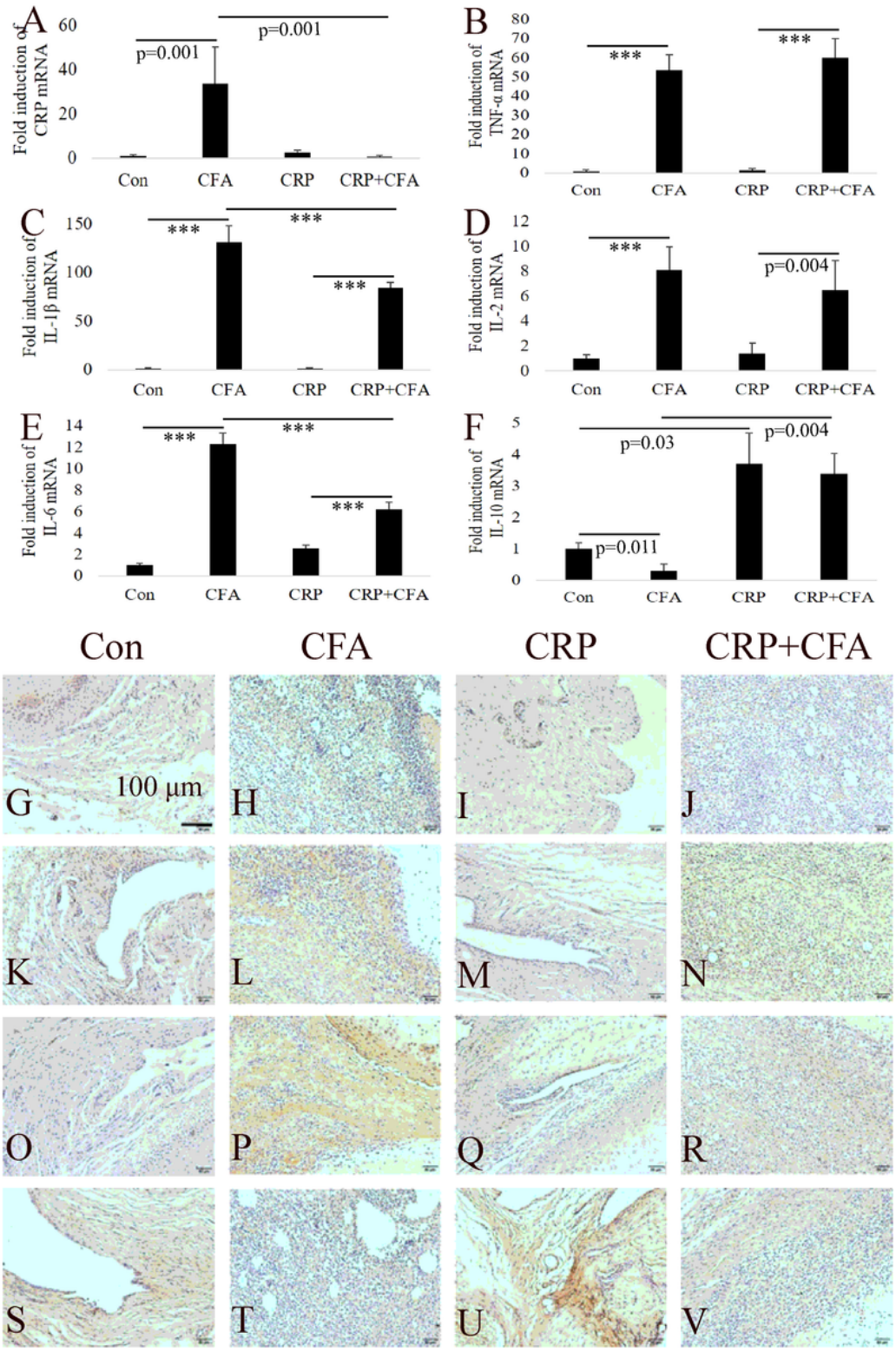
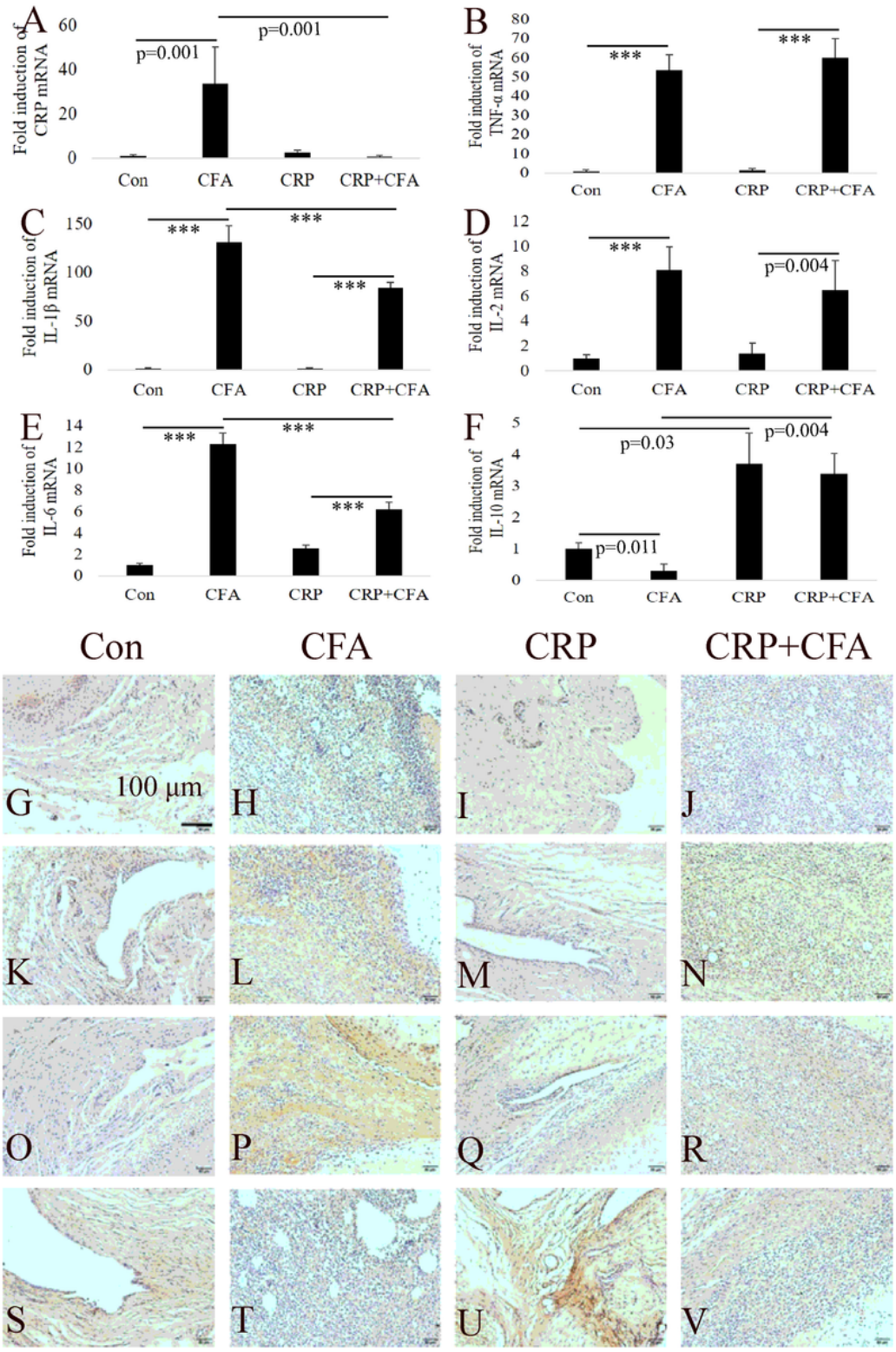


Figure 4

Inflammatory cytokines expression based on PCR and IHC results The mRNA expression level of (A) CRP, (B) TNF-α, (C) IL-1β, (D) IL-2, (E) IL-6, and (F) IL-10. (N=8; mean+SD; ***p < 0.001). (G-J) The IHC results of CRP expression in synovial membrane among groups. (K-N) The IHC results of TNF-α among groups. (O-R) The IHC results of IL-1β among groups. (S-V) The IHC results of IL-10 among groups.



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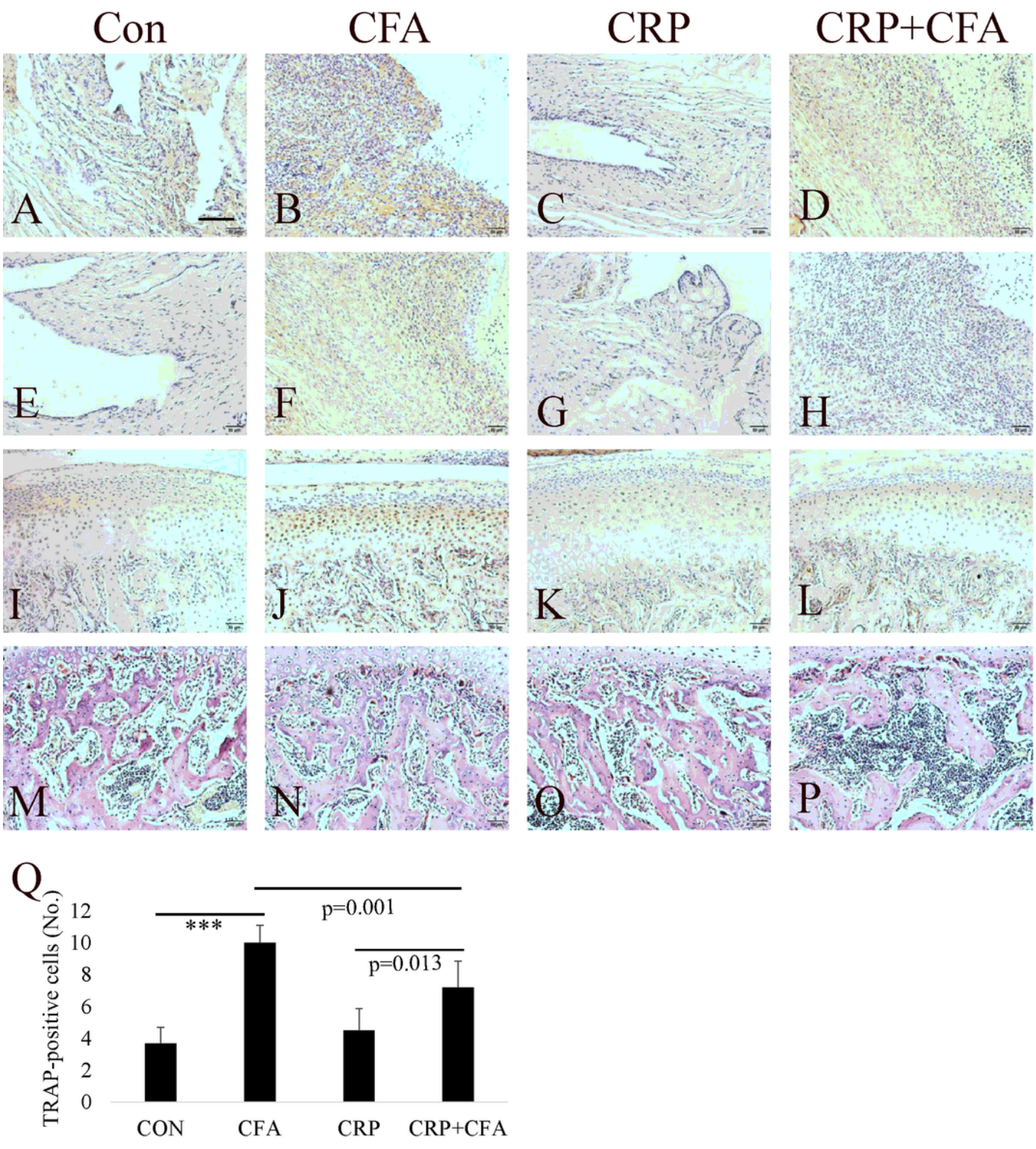
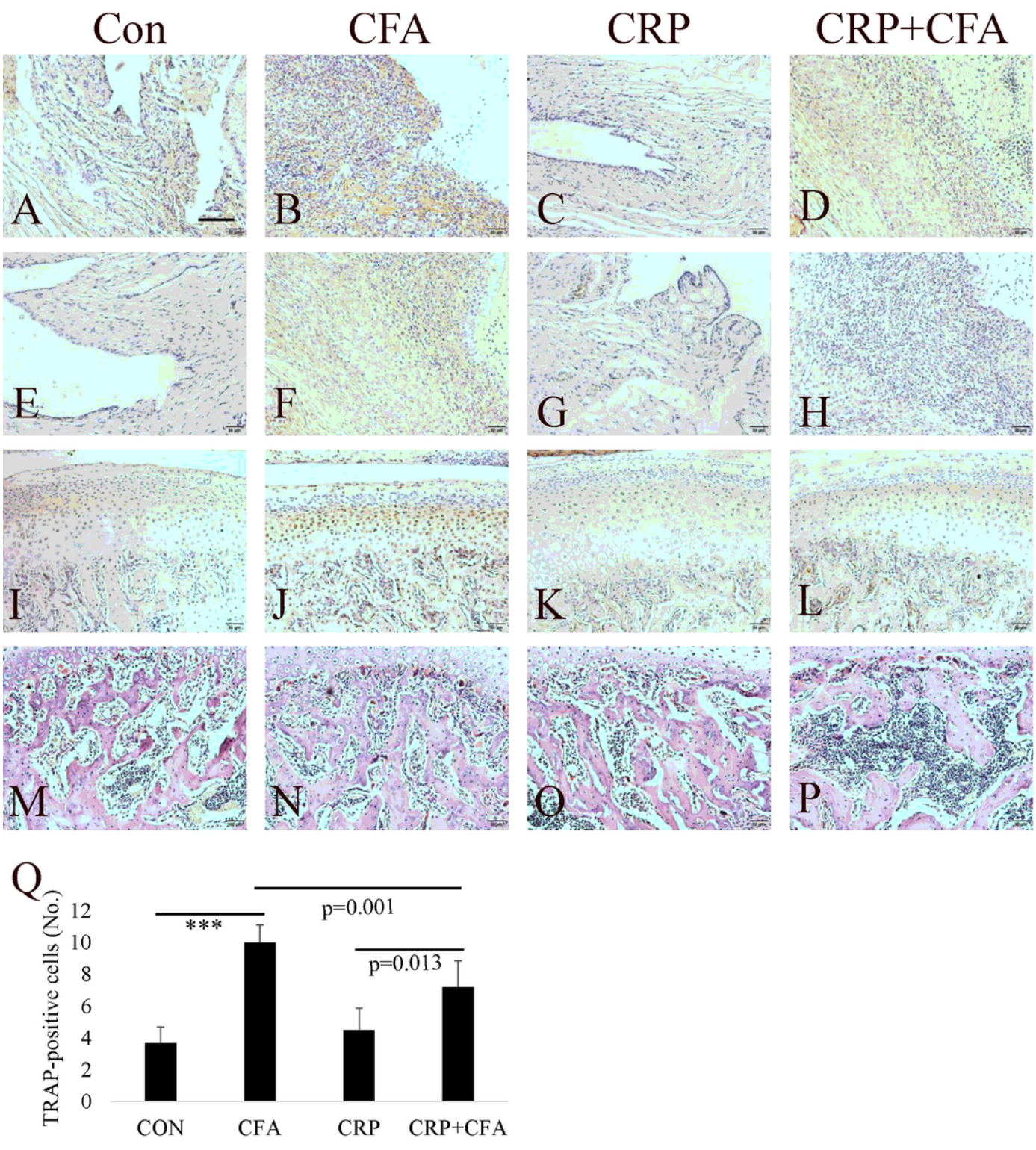


Figure 5

Macrophage and osteoclast activity with photomicrographs of immunopositive cells in synovium (A-D) The IHC results of CD68 expression in synovial membrane among groups. (E-H) The IHC results of iNOS among groups. (I-L) The IHC results of RANKL expression in TMJ cartilage among groups. (Q) Statistical result of TRAP-positive cells (cluster of more than 3 nuclei was counted as one osteoclast) among groups. (N=6; mean+SD; ***p < 0.001).



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Supplementary Files

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- Supplementarymaterials.docx
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