의학석사 학위논문

Effect of Hydroxychloroquine Treatment on Dry Eyes in subjects with Primary Sjögren's Syndrome: double blind randomized control study

1차 쇼그렌 증후군 환자의 건성안에 대한 Hydroxychloroquine의 치료 효과: 이중맹검 연구

2014년 2월

서울대학교 대학원

임상의과학과

윤 창 호

1차 쇼그렌 증후군 환자의 건성안에 대한 Hydroxychloroquine의 치료 효과: 이중맹검 연구

Effect of Hydroxychloroquine Treatment on Dry Eyes in subjects with Primary Sjögren's Syndrome: double blind randomized control study

February 2014

Department of Clinical Medical Sciences

Seoul National University

College of Medicine

Chang Ho Yoon

Effect of Hydroxychloroquine Treatment on Dry Eyes in subjects with Primary Sjögren's Syndrome: double blind randomized control study

지도 교수 김 미 금

이 논문을 의학석사 학위논문으로 제출함

2013년 10월

서울대학교 대학원

임상의과학과

윤 창 호 **(Chang Ho Yoon)**

윤창호의 의학석사 학위논문을 인준함

2014년 1월

위 원 장 위 원 량 (인)

부위원장 김 미 금 (인)

위 원 허 장 원 (인)

Abstract

Introduction: Effect of hydroxychloroquine (HCQ) on dry eye has not been fully determined yet. This study was aimed to compare the 12-weeks efficacy of HCQ medication with placebo in the management of dry eye in Primary Sjögren's Syndrome (pSS).

Methods: A double-blind, randomised control study was conducted in 39 subjects of 153 pSS subjects from May 2011 through August 2013. The diagnosis of Sjögren's syndrome was made on the basis of the classification criteria of the American-European Consensus Group. Subjects received 300 mg HCQ or placebo once daily for 12 weeks. Subjects were evaluated at baseline, 6 weeks and 12 weeks and re-visited at 16 weeks after drug discontinuance. Fluorescein staining score, Schirmer test score, tear film break-up time (TBUT), and ocular surface disease index (OSDI) were measured, and tears and blood were collected for ESR, IL-6, IL-17, B-cell activating factor (BAFF) and Th17 cell analysis. Color test and fundus were examined to monitor HCQ complications.

Results: Twenty-six subjects completed follow-ups. Fluorescein staining score and Schirmer test score did not differ significantly. The OSDI improved

with medication, but this was not insignificant between the groups. TBUT, Serum IL-6, ESR, serum and tear BAFF, and the proportion of Th17 cells did not change in either group.

Conclusions: HCQ at 300 mg daily has no apparent clinical benefit on dry eye and systemic inflammation in pSS.

Keywords: Hydroxychloroquine, Sjögren's Syndrome, Double blind randomized prospective study, OSDI

Student Number: 2011-22002

TABLE OF CONTENTS

LISTS of TABLE and FIGURES

Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease involving lacrimal and salivary glands with resultant keratoconjunctivitis sicca and xerostomia, and lymphocytic infiltration of exocrine glands and epithelium is a common pathological finding [1]. It is one of the most prevalent rheumatologic diseases and has female predominance [2]. About one-third of SS patients have extraglandular systemic involvement, and SS patients with or without other autoimmune rheumatic disease are defined as secondary SS (sSS) or primary SS (pSS), respectively. In 2002, the American–European Consensus Group defined the rules for classifying between pSS and sSS mainly by using a serologic marker (anti-Ro/La antibody level) and histopathology of salivary glands [3].

Regarding dry eye in SS, conjunctival and corneal staining tests, the Schirmer test, and symptoms are worse in the patients with than without SS among aqueous-deficient dry eye patients [4]. Conjunctivocorneal epithelial disintegration results in blurred vision, severe discomfort, and increased risk of infection. Therefore, severe dry eye in SS patients results in an ongoing poor quality of life.

The first treatment option of dry eye symptoms is topical drugs such as artificial tears or cyclosporine A [2, 5]. However, topical treatment is not usually sufficient for severe dry eye in pSS patients. Because inflammatory markers are increased in SS patients, most systemic medications are aimed at

immunologic pathways [6]. Antimalarial drugs are frequently prescribed to SS patients as non-specific blockers of toll-like receptor 9/7 (TLR9/TLR7), but their effectiveness in dry eye is controversial. Improvement in immunologic markers such as IL-6, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), and B-cell activating factor (BAFF), as well as sicca signs such as the tear break-up test (TBUT) and Schirmer test, has been reported in a few studies after oral management with hydroxychloroquine (HCQ) [7, 8]. However, there is only one two-year double-blind crossover trial for pSS in 19 patients, which demonstrated that HCQ treatment does not have clinical benefits for pSS patients [9].

Therefore, to confirm the effect of oral HCQ treatment in dry eye in pSS, we conducted a prospective randomised, double-blind trial comparing HCQ treatment with a placebo control.

Patients and methods

This randomised controlled trial was conducted at the Department of Ophthalmology at Seoul National University Hospital from May 2011 through August 2013, and was registered with ClinicalTrials.gov (Identifier: NCT01601028). Written informed consent was obtained from all subjects, and the study was granted ethical approval by the institutional review board of Seoul National University Hospital (IRB number: H-1104-083-359). This study was conducted in accordance with the tenets of the Declaration of Helsinki.

Patients

Inclusion criteria were (1) adult (aged >18 years) patients with pSS diagnosed on the basis of the 2002 American–European Consensus Group (AECG) criteria [3] and (2) subjects who had the ability to give informed, dated, and signed consent before the beginning of any proceedings related to the trial.

Exclusion criteria were (1) previous treatment with HCQ with insufficient discontinuation time (3 weeks) after discontinuance of treatment; (2) known cardiac disease, respiratory disease, renal disease, or gastrointestinal disease (except gastrooesophageal reflux disease); (3) diabetes mellitus; (4) psoriasis; (5) known drug allergy or hypersensitivity; (6) previous or ongoing treatment

with any other drugs (including topical drugs) that might affect the lacrimal system with insufficient washout time after discontinuance of treatment (e.g. SSRI, anti-histamines, and pilocarpine); (7) closed-angle glaucoma; (8) previous intraocular surgery; (9) macular disease; (10) previous or ongoing treatment with drugs that might have an effect on the macula; (11) pregnancy; and (12) planning pregnancy.

A double-blind, randomised control study was conducted in 39 subjects of 153 pSS subjects for 2 years. We were unable to recruit the intended 60 subjects within the 2-year recruitment period because many subjects with pSS did not meet our inclusion criteria. Most of the subjects did not wish to discontinue previous oral drugs because of severe systemic symptoms. During the study period, 153 pSS patients visited our clinic. Among them, only 67 subjects met all eligibility criteria. Twenty-eight subjects declined to participate in the trial, and 39 subjects were finally enrolled in this study (Figure 1).

During the study period of 2 years, a new criterion of SS was reported [10]. Because this study was designed before the recent criterion was published, we enrolled subjects according to the previous criteria of the American–European Consensus Group to maintain consistency of the subject population.

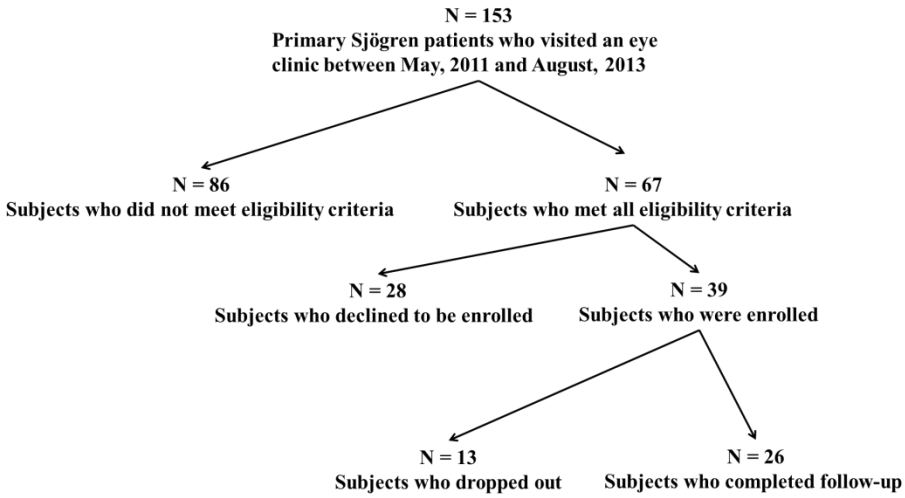

Figure 1. A double-blind randomized control study was conducted in 39 subjects of 153 pSS subjects who visited an eye clinic from May 2011 through August 2013.

Treatment protocol

All subjects underwent initial medical and ophthalmologic history taking and physical examination at the baseline visit (week 0). The study eye was selected as the one which showed the greater corneal fluorescein staining score (on a scale of 0–15; National Eye Institute scale [11] at the baseline visit (Figure 2). Subjects were randomly assigned to the HCQ or placebo group by using sealed randomisation envelopes. The placebo was manufactured identical in appearance to the active drug, and all tablets were kindly supplied by Kyung Poong Pharma Co. Both investigators and subjects were blinded to the treatment assignments. Subjects were instructed to take two tablets (one 200 mg tablet and one 100 mg tablet) of the study medication, giving a total of 300 mg, once daily (qd) for 12 weeks and to report missed doses and adverse events. All subjects used Hyalein Mini ophthalmic solution 0.1% (hyaluronic acid 0.1%; Santen, Osaka, Japan) six times per day. Other topical medications were restricted. Participants returned to the study site at 6 and 12 weeks for efficacy and safety evaluations. After 12 weeks, oral HCQ and placebo medication were withheld, while topical medication was continued in all subjects for 4 weeks. Subjects revisited the clinic at 16 weeks. Schematic schedule arrangement was shown in Figure 3. At each visit, visual acuity, corneal fluorescein staining score using NEI grading, Schirmer test without anaesthesia, tear film break-up time (TBUT), Hardy Rand and Rittler (HRR) colour vision test, and fundus examination were performed. Subjective assessment using the ocular surface disease index questionnaire (OSDI) [12]

was conducted. The OSDI questionnaire was translated to Korean versions from the original English. Tear and blood samples were collected for measurement of IL-6, BAFF, ESR, IL-17, and Th17 cells which secrete IL-17.

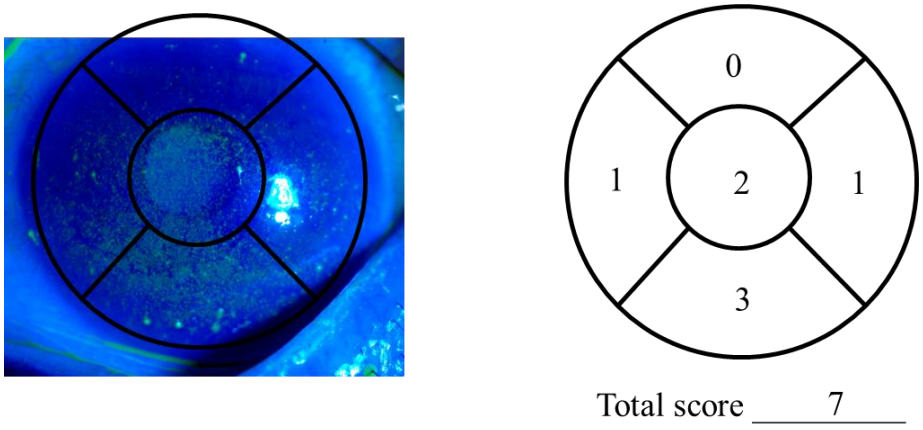

Figure 2. Corneal fluorescein staining score using the National Eye Institute scale (on a scale of 0–15) in a primary Sjögren's syndrome subject

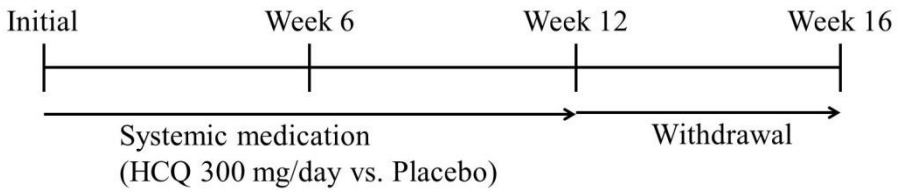

Figure 3. Schematic design of the study protocol regarding time-dependent treatment and examinations

Tear sample collection

Tear fluid was obtained from patients at the same time at baseline and 6, 12, and 16 weeks Tears were collected from the medial and lateral canthus. Topical anaesthesia was not used. To minimise ocular surface irritation, we obtained tear samples by using a Merocel sponge (PVA 0525; Oasis, Glendora, CA, USA) [13]. After collection, the sponge was inserted into a 0.5-mL tube (Eppendorf, Fremont, CA, USA) and the tear fluid was subsequently recovered by centrifugation at 10,000 rpm for 10 min.

Measurement of tear and serum cytokine profiles

Serum ESR was measured by conventional methods in our hospital laboratory on the day of the patient visit. For BAFF and IL-6, 0.5 mL plasma was separated from heparinised peripheral blood and stored together with the tear sample at -70℃ until further examination.

Concentrations of BAFF, IL-6, and IL-17A were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. ELISA using serum was performed in duplicate to ensure the reproducibility of the data. However, ELISA using tears was performed once because of the lack of significant amounts of tears. Tear samples were diluted 1:12.5 and 1:100 for BAFF and IL-17, respectively.

The concentration ranges used for the standard curve were 62.5–4000 pg/mL,

9.38–600 pg/mL, and 15.6–1,000 pg/mL of human recombinant BAFF, IL-6, and IL-17, respectively (R&D Systems).

Flow cytometric analysis for Th17 cells

Flow cytometry was also performed on the same day as the patient visit. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation (BIOCHROM Inc., Cambridge, UK). PBMC were stimulated for 6 h with 50 ng/mL 1-phorbol-12-myristate-13-acetate (PMA; Sigma-A Aldrich, St. Louis, MO, USA) and 1 μg/mL ionomycin (Sigma-A aldrich) in the presence of brefeldin A (BD Bioscience, San Jose, CA, USA) for the final 4 h. The cells were fixed and permeabilised using a BD Cytofix/Cytoperm™ kit (BD Bioscience). The fixed cells were stained with anti-CD3-APC-Cy7 and anti-CD4-FITC for 30 min at 4℃ for initial surface staining. For intracellular staining, they were incubated with anti-IFN-γ-PE-Cy7 (all from BD Bioscience) and anti-IL-17A-PE (eBioscience, SanDiego, CA) mAb for 60 min at 4℃. Flow cytometric analysis was performed using a FACS BD LSR II (BD Bioscience), and the data were analysed by FlowJo (Treestar, Ashland, OR, USA). Isotype mouse Ig G1-PE-Cy7 (eBioscience) and mouse Ig G1- PE (eBioscience) were used for control.

After four-colour compensation, lymphocytes were gated in the forward scatter (FSC)/side scatter (SSC) gate, and CD4 T cells were then gated by

$CD3^{hi}$ and $CD4^{hi}$ expression; finally, the cells were gated by IFNγ and IL17 (Figure 4). The percentage of Th17 cells was calculated by summating $IL17^{hi}$ cells and IFNγ/IL17 $^{double\ hi}$ cells.

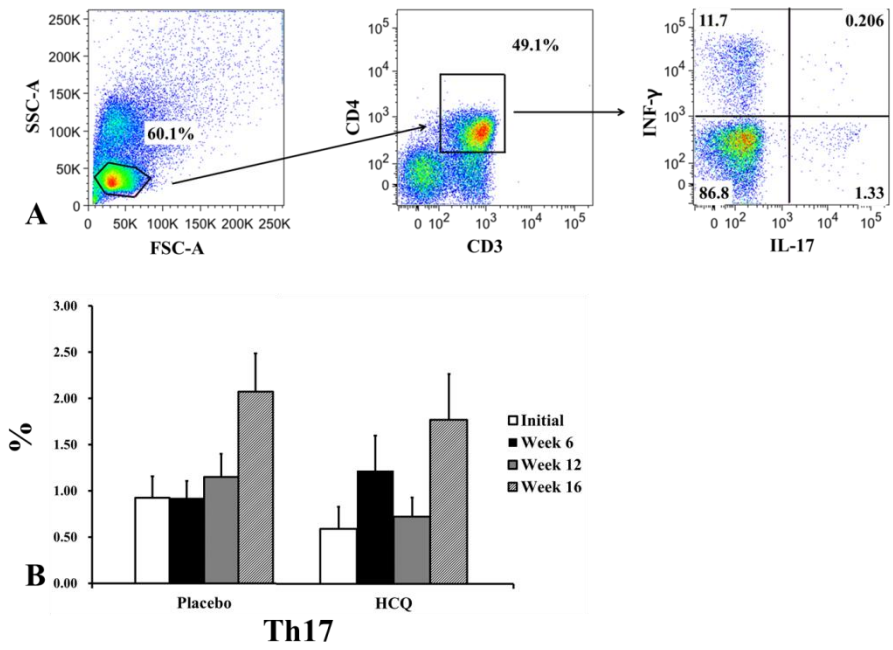

Figure 4. Flow cytometric analysis of Th17 cells which were gated by $CD3^{hi}/CD4^{hi}$ lymphocytes and then gated by IFNγ/IL-17 (A), and time-dependent percent changes of the Th17 cells between the groups (B). Bars represent the mean + standard error.

Sample size calculations

A previous literature review on SS did not include results of tear IL-17 concentration after HCQ medication. Tear IL-17 concentration of SS patients was 504.91 pg/mL and 352.45 pg/mL in the total and mild keratoconjunctivitis sicca groups, respectively, according to this previous report [14]. We assumed the tear IL-17 concentration after HCQ medication would be similar to that of the mild keratoconjunctivitis sicca group. A sample size of 60 patients (30 in each group) was needed to detect 504.91 ± 136.38 pg/mL versus 352.45 ± 136.38 pg/mL between the 2 groups using a 2-sided test with a power of 80% with the significance level controlled at 5%. Sample size calculation was assisted by the Medical Research Collaborating Center of Seoul National University Hospital.

Statistical analysis

Primary analyses of data were based on the per-protocol population, which included all participants who took the 12-week study medication. Statistical analyses were performed using PASW software for Windows (v. 19.0; SPSS Inc., Chicago, IL, USA). A p value of <0.05 was considered statistically significant. The Shapiro–Wilk test was applied to evaluate the data normality. Two groups were compared with a Student's *t*-test (two-tailed) and a Mann–Whitney *U* test according to the normality test result. Differences between the HCQ and placebo groups during the course of the treatment were analysed by

repeated-measures ANOVA and generalized estimating equation when there were non-normality or missing data.

Results

General complications of the subjects and characteristics of drop-out subjects

Of the 39 recruited subjects, 26 were treated per protocol. Thirteen subjects including five in the placebo group and nine in the HCQ group did not complete the study. In the placebo group, three subjects did not keep their follow-up appointments and were unavailable to be reached, one subject suffered dyspepsia, and one subject was found to be unsatisfactory for enrolment. In the HCQ group, two subjects did not keep their follow-up appointments and were unable to be contacted, three subjects suffered dyspepsia, one subject did not have the time for follow-up examinations, two subjects were found deficient in enrolment criteria, and one subject developed incidental subretinal haemorrhage due to occult myopic choroidal neovascularisation (CNV) with accompanying impaired vision 3 days after medication, which was not regarded as a drug-related complication. There were no other drug-related side effects regarding ocular complications (optic neuropathy or maculopathy) or general subjective complications except dyspepsia (10.3%).

Characteristics of the study population at baseline

Of the 26 pSS subjects analysed, 11 subjects were randomised to

receive HCQ and the remaining 15 subjects were randomised to receive placebo. All subjects were women with a mean age of 56.85 years (range, 35–74). The mean (range) age was 55.0 (35–73) years in the placebo group and 59.4 (44–74) years in the HCQ group, which was not significantly different ($p = 0.263$, independent *t*-test). Initial results of serum ESR, IL-6, BAFF, Th17, tear BAFF, TBUT, Schirmer score, corneal staining score, and OSDI are shown in Table 1. There were no significant differences between the groups in all of the demographic factors and parameters.

Changes in the parameters

Changes in parameters are shown in Table 2 and Figures 4 and 5. Regarding systemic inflammatory parameters, mean ESR tended to continuously increase in the placebo group and decrease in the HCQ group, although there were no significant differences between the groups. Mean serum IL-6, BAFF, proportion of Th17 cells, tear BAFF and BUT did not show statistically significant differences between the groups. Tear IL-17 did not detect almost of the samples.

Table 1. Demographics and initial characteristics of the subjects

	Total (N = 26)	Placebo (n = 15)	HCQ (n = 11)	P
Age (yr)	56.85 ± 9.66	55.0 ± 9.72	59.4 ± 9.42	0.263*
Serum ESR (mm/h)	23.42 ± 14.78	19.67 ± 12.08	28.55 ± 17.08	0.133*
Serum IL-6 (pg/ml)	4.31 ± 7.45	4.20 ± 4.90	4.45 ± 10.25	0.134†
Th17 (%)	0.80 ± 0.74	0.93 ± 0.80	0.61 ± 0.70	0.340†
Serum BAFF (pg/ml)	2300 ± 859	2327 ± 1028	2261 ± 601	0.878†
Tear BAFF (pg/ml)	384 ± 220	436 ± 328	417 ± 184	1.000†
TBUT (sec)	2.52 ± 0.85	2.23 ± 0.63	2.78 ± 1.09	0.439†
Schirmer (mm)	4.12 ± 3.90	4.00 ± 3.07	4.27 ± 4.98	0.878†
Corneal Staining Score	3.27 ± 1.71	3.33 ± 1.54	3.18 ± 1.99	0.829*
OSDI	47.92 ± 28.30	43.40 ± 27.33	54.08 ± 29.74	0.352*

*Independent t test, †Mann–Whitney U test

Abbreviations: HCQ, hydroxychloroquine; IL, Interleukin; Th17, T helper 17 cells; BAFF, B-cell activating factor; TBUT, tear film break-up time; OSDI, Ocular Surface Disease Index

Table 2. Changes of the parameters between the HCQ and placebo groups during follow-up

			Baseline	Week 6	Week 12	Week 16	P (baseline - week 12)	P (week 12 vs. 16)
Parameter	ESR (mm/hr)	Placebo	19.67 ± 12.08	20.13 ± 8.12	21.40 ± 11.38	20.20 ± 12.52	0.571*	0.620*
		HCQ	28.55 ± 17.08	27.36 ± 18.49	24.91 ± 20.94	21.09 ± 12.09		
	IL-6 (pg/ml)	Placebo	4.20 ± 4.90	5.31 ± 8.35	5.01 ± 6.83	5.83 ± 8.37	0.451†	0.991*
		HCQ	4.45 ± 10.25	4.77 ± 9.21	5.37 ± 10.32	6.34 ± 14.14		
	Th17 (%)	Placebo	0.93 ± 0.80	1.05 ± 0.73	1.14 ± 0.98	2.07 ± 1.48	0.199†	0.566*
		HCQ	0.61 ± 0.70	1.05 ± 1.32	0.91 ± 0.77	1.79 ± 1.65		
	Serum BAFF (pg/ml)	Placebo	2327 ± 1028	2513 ± 1903	2480 ± 1410	N/A	0.340†	N/A
		HCQ	2261 ± 601	2071 ± 492	2116 ± 711			
	Tear BAFF (pg/ml)	Placebo	436 ± 328	141 ± 56	427 ± 256	N/A	0.723†	N/A
		HCQ	417 ± 184	1094 ± 1418	1113 ± 1466			
	TBUT (sec)	Placebo	2.23 ± 0.63	2.60 ± 0.83	2.87 ± 0.99	2.57 ± 0.76	0.125†	0.746*
		HCQ	2.78 ± 1.09	2.60 ± 0.84	2.45 ± 0.52	2.18 ± 0.40		
	Schirmer (mm)	Placebo	4.00 ± 3.07	4.33 ± 4.22	3.20 ± 2.68	3.43 ± 2.14	0.136†	0.958*
		HCQ	4.27 ± 4.98	2.50 ± 1.08	2.82 ± 2.40	2.91 ± 2.26		
	Corneal Staining Score	Placebo	3.33 ± 1.54	3.20 ± 2.01	3.67 ± 1.54	4.07 ± 2.09	0.128†	0.524*
		HCQ	3.18 ± 1.99	3.10 ± 2.18	2.54 ± 2.16	2.54 ± 2.42		
	OSDI	Placebo	43.40 ± 27.33	32.45 ± 19.21	30.47 ± 23.47	30.92 ± 28.17	0.209*	0.292*
		HCQ	54.08 ± 29.74	30.37 ± 28.63	22.88 ± 21.51	27.75 ± 21.73		

*Repeated-measures ANOVA, †Linear mixed model

Abbreviations: HCQ, hydroxychloroquine; IL, Interleukin; Th17, T helper 17 cells; BAFF, B-cell activating factor; TBUT, tear film break-up time; OSDI, Ocular Surface Disease Index

With respect to the ocular signs and symptoms, TBUT did not change significantly from baseline to week 12 in either group. The Schirmer test score showed no statistical changes in either group. In corneal staining score, the HCQ group tended to show continuous reduction of the score, although it was not significant. The OSDI was significantly decreased between baseline and weeks 6 and 12 (baseline vs. 6 weeks, $p = 0.024$; baseline vs. 12 weeks, $p = 0.02$; repeated-measures ANOVA with Bonferroni adjustment), suggesting improvement of subjective symptoms. However, there was no statistically significant difference between the HCQ and placebo groups ($p = 0.209$, repeated-measures ANOVA).

After discontinuation of the oral medication, only the proportion of Th17 cells was significantly increased in both groups ($p < 0.01$, repeated-measures ANOVA). However, there was no difference between the groups ($p = 0.566$). Other parameters did not show significant changes after oral medications were discontinued (Table 2).

Discussion

No definite beneficial effect of the use of HCQ in the treatment of dry eye in pSS was found in this study, although there was evidence of improved subjective ocular symptoms. Study results failed to show that HCQ treatment might affect tear production and inflammatory parameters such as ESR, IL-6, BAFF, and Th17 cells in pSS patients

The main anti-inflammatory mechanism of HCQ is considered non-specific antagonism at TLR9 and TLR7 [15, 16]. Circulating DNA- and RNA-containing immune complexes in the blood may stimulate plasmacytoid dendritic cells (pDCs) through TLR9 and TLR7 [16, 17]. Activated pDCs produce IL-6, which can induce co-stimulatory molecules, and stimulated co-stimulatory molecules combined with T cell receptor activate T helper (Th) cells [18, 19]. Recent study has revealed that Th17 cells are up-regulated and involved in the main pathogenic pathways in RA, SS, SLE, and GVHD [20, 21] and many studies have shown that IL-17 level is increased in serum, saliva, and tears in SS patient [14, 21, 22]. In addition, activated pDCs interact with B cells, which produce BAFF. BAFF is an essential homeostatic cytokine for B cells that regulate both innate and adaptive immunity [23]. IL-6 and BAFF are over-expressed in SS patients [24]. The lack of tear production in the SS subjects limited our selection of inflammatory

parameters. Therefore, we analysed IL-6, Th17 cells, BAFF, and ESR in serum and BAFF and IL17 in tears.

We evaluated the effect of HCQ on dry eye as well as systemic changes of inflammatory parameters. Serum and tear BAFF were also not significantly changed. There are two previous reports about BAFF after HCQ treatment: serum BAFF was decreased after HCQ medication; tear BAFF level increased after HCQ discontinuation [8, 25]. But patients of these two reports were constantly took oral HCQ mediation for more than 2 years before enrollment. This could make bias that only patients who was better after HCQ medication were remain at the time of enrollment. Serum ESR, and IL-6 were not significantly changed, which was comparable with previous study [9]. However, Tishler et al. [7] reported that serum ESR and IL-6 were significantly decreased after 12 months, but not during 6 months, of HCQ treatment (200 mg daily). Possible reasons for the lack of changes in ESR and IL-6 are as follows: (1) HCQ might have no clinically beneficial anti-inflammatory effect. (2) Short-term usage (12 weeks) might have been inadequate to stop the chronic inflammatory process and vicious cycle although effective drug concentration is reached in the serum within 2 weeks. (3) Previous studies of HCQ in SS patients permitted low dose of oral steroid or topical cyclosporine A [8, 26]. Our

patients were not allowed any other oral or topical anti-inflammatory medication, which might have caused the results in our study to differ from those of the other studies. (4) The small number of enrolled subjects might have affected statistical significance. Disappointingly, HCQ showed no definite effect on Th17 cells. Possible reasons are as mentioned above. In addition, the dosage of 300 mg daily might not be sufficient to downgrade aggravated inflammation such as activated Th17 cells.

Regarding ocular changes, the Schirmer test score and BUT did not change significantly during follow-up after HCQ treatment and showed no difference between the groups. This suggests that HCQ treatment did not affect the tear production in this study. The presumed reasons for this finding are as follows: (1) HCQ actually has no clinically beneficial anti-inflammatory effect. (2) Considering the age of the subjects (mean age, 56.8 years), most of the lacrimal gland might have been destroyed by chronic inflammation before treatment. HCQ treatment might affect tear production in younger study subjects with early inflammation. HCQ treatment showed time-dependent significant improvement of subjective ocular symptoms in the HCQ group, although there were no significant differences between the HCQ and placebo groups.

Subjective symptoms represented by OSDI were improved while taking the medication, but this was insignificant between the groups. The improved effect may be because of the topical artificial tear or placebo effect. Moreover, the small group numbers might explain why there was no difference between the two groups.

Taken together, HCQ medication did not show significant difference in pSS during the study period. But we did not investigate all of the anti-inflammatory cytokines and inflammatory cells because of the small quantities of collected tears and blood. We might have missed some other anti-inflammatory function of HCQ.

There were several limitations in this study. First, appropriate sample size of the subjects to draw statistical significance could not be achieved. Many of the pSS patients were already taking oral medications such as HCQ, pilocarpine, steroid, or other immune suppressants and were reluctant to stop the medicine for a sufficient wash-out period. Further, budget constraints prevented prolonging the study to enrol the targeted sample number. Second, the study schedule was short. Previous studies which reported significant positive results of HCQ in pSS ran for 12 months, [7, 9] or included cessation of 3 months after 48 months or more of treatment [8]. Third, the small number of enrolled patients might have affected significant differences.

Therefore, further large-scale prospective study is warranted. Nevertheless, our study is worthy of notice because it supports other reports and builds up evidence that HCQ does not have a definite beneficial effect on dry eye or systemic inflammation.

In conclusion, HCQ treatment at a dose of 300 mg daily does not appear to have a clinical benefit on dry eye and systemic inflammation in subjects with pSS.

Acknowledgement

This study was supported by a grant from the Seoul National University Hospital (Project No. 0420110480). Kyung Poong Pharma Co. kindly provided tablets of hydroxychloroquine and placebo.

References

1. Wu CH, Hsieh SC, Lee KL, et al. Pilocarpine hydrochloride for the treatment of xerostomia in patients with Sjogren's syndrome in Taiwan--a double-blind, placebo-controlled trial. J Formos Med Assoc 2006;105(10):796-803.

2. Ramos-Casals M, Tzioufas AG, Stone JH, et al. Treatment of primary Sjogren syndrome: a systematic review. JAMA 2010;304(4):452-60.

3. Vitali C, Bombardieri S, Jonsson R, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61(6):554-8.

4. Liew MS, Zhang M, Kim E, Akpek EK. Prevalence and predictors of Sjogren's syndrome in a prospective cohort of patients with aqueous-deficient dry eye. Br J Ophthalmol 2012;96(12):1498-503.

5. Sall KN, Cohen SM, Christensen MT, Stein JM. An evaluation of the efficacy of a cyclosporine-based dry eye therapy when used with marketed artificial tears as supportive therapy in dry eye. Eye Contact Lens 2006;32(1):21-6.

6. Becker H, Pavenstaedt H, Willeke P. Emerging treatment strategies and potential therapeutic targets in primary Sjogren's syndrome. Inflamm Allergy Drug Targets 2010;9(1):10-9.

7. Tishler M, Yaron I, Shirazi I, Yaron M. Hydroxychloroquine treatment for primary Sjogren's syndrome: its effect on salivary and serum inflammatory markers. Ann Rheum Dis 1999;58(4):253-6.

8. Yavuz S, Asfuroglu E, Bicakcigil M, Toker E. Hydroxychloroquine improves dry eye symptoms of patients with primary Sjogren's syndrome. Rheumatol Int 2011;31(8):1045-9.

9. Kruize AA, Hene RJ, Kallenberg CG, et al. Hydroxychloroquine treatment for primary Sjogren's syndrome: a two year double blind crossover trial. Ann Rheum Dis 1993;52(5):360-4.

10. Shiboski SC, Shiboski CH, Criswell L, et al. American College of Rheumatology classification criteria for Sjogren's syndrome: a data-driven, expert consensus approach in the Sjogren's International Collaborative Clinical Alliance cohort. Arthritis Care Res (Hoboken) 2012;64(4):475-87.

11. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. CLAO J 1995;21(4):221-32.

12. Schiffman RM, Christianson MD, Jacobsen G, et al. Reliability and validity of the Ocular Surface Disease Index. Arch Ophthalmol 2000;118(5):615-21.

13. Acera A, Rocha G, Vecino E, et al. Inflammatory markers in the tears of patients with ocular surface disease. Ophthalmic Res 2008;40(6):315-21.

14. Chung JK, Kim MK, Wee WR. Prognostic factors for the clinical severity of keratoconjunctivitis sicca in patients with Sjogren's syndrome. Br J Ophthalmol 2012;96(2):240-5.

15. da Silva JC, Mariz HA, da Rocha LF, Jr., et al. Hydroxychloroquine decreases Th17-related cytokines in systemic lupus erythematosus and rheumatoid arthritis patients. Clinics (Sao Paulo) 2013;68(6):766-71.

16. Kuznik A, Bencina M, Svajger U, et al. Mechanism of endosomal TLR

inhibition by antimalarial drugs and imidazoquinolines. J Immunol 2011;186(8):4794-804.

17. Seillet C, Laffont S, Tremollieres F, et al. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor alpha signaling. Blood 2012;119(2):454-64.

18. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Am J Pathol 2012;181(1):8-18.

19. Mills KH. TLR9 turns the tide on Treg cells. Immunity 2008;29(4):518-20.

20. Katsifis GE, Rekka S, Moutsopoulos NM, et al. Systemic and local interleukin-17 and linked cytokines associated with Sjogren's syndrome immunopathogenesis. Am J Pathol 2009;175(3):1167-77.

21. Oh JY, Kim MK, Choi HJ, et al. Investigating the relationship between serum interleukin-17 levels and systemic immune-mediated disease in patients with dry eye syndrome. Korean J Ophthalmol 2011;25(2):73-6.

22. Nguyen CQ, Hu MH, Li Y, et al. Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjogren's syndrome: findings in humans and mice. Arthritis Rheum 2008;58(3):734-43.

23. Moisini I, Davidson A. BAFF: a local and systemic target in autoimmune diseases. Clin Exp Immunol 2009;158(2):155-63.

24. Roescher N, Tak PP, Illei GG. Cytokines in Sjogren's syndrome: potential therapeutic targets. Ann Rheum Dis 2010;69(6):945-8.

25. Mumcu G, Bicakcigil M, Yilmaz N, et al. Salivary and Serum B-cell

Activating Factor (BAFF) Levels after Hydroxychloroquine Treatment in Primary Sjogren's Syndrome. Oral Health Prev Dent 2013;11(3):229-34.

26. Fox RI, Dixon R, Guarrasi V, Krubel S. Treatment of primary Sjogren's syndrome with hydroxychloroquine: a retrospective, open-label study. Lupus 1996;5 Suppl 1:S31-6.

국 문 초 록

서론: Hydroxychloroquine (HCQ)은 쇼그렌 증후군 환자에게 오래 전부터 사용되어 온 약으로 아직 기전에 대한 것은 잘 밝혀져 있지 않다. 본 연구는 1차 쇼그렌 환자에서 12주간의 HCQ의 복용 효과를 전향적 무작위 이중 맹검법을 이용한 위약 대조군 연구방법을 이용하여 확인하고자 하였다.

방법: 2011년 5월부터 2013년 8월까지 내원한 153명의 1차 쇼그렌 환자 중 39명을 대상으로 이중맹검 위약-대조군 시험을 시행하였다. 1차 쇼그렌 증후군 환자의 진단은 American-European Consensus Group의 진단기준을 사용하였다. 환자군을 하루 300 mg 의 HCQ 복용군과 위약군으로 나누었다. 환자는 복용 시작 직전, 복용 6주째, 복용 12주째 그리고 복용을 4주간 중단 후 내원하였다. 내원시마다 각막염색검사, 쉬머검사, 눈물막 파괴시간, 안구표면질환지수 (OSDI)를 측정하였고, 혈액의 ESR, IL-6, B-cell activating factor (BAFF), Th17세포, 눈물의 BAFF, IL-17의 검사를 위해 내원시마다 혈액과 눈물을 채취하였다. 한편 망막 독성여부를 보기 위해서 색각검사, 안저검사를 내원시마다 검진하였다.

결과: 26명의 환자가 최종적으로 모집되었다. 각막염색수치 및 쉬머 검사는 두 군간의 차이가 없었다. 반복측정분산분석으로 분석시 안구표면질환지수는 26명의 환자에서 유의하게 호전되었으나 (P = 0.02) 위약군과 차이가 없었다 (p = 0.209). 눈물막 파괴시간, 혈액의 IL-6, ESR, BAFF, Th17 세포의 비율은 차이 없었고 눈물의 BAFF역시 두 군에서 모두 치료기간 동안 차이를 보이지 않았다.

결론: 1차 쇼그렌증후군 환자에서 하루 300 mg의 HCQ복용은 각막건조증과 전신 염증상태의 호전에 이득이 없었다.

주요어: hydroxychloroquine, 쇼그렌 증후군, 전향적 무작위 이중 맹검법, 안구표면질환지수

학　번: 2011 – 22002

의학석사 학위논문

Effect of Hydroxychloroquine Treatment on Dry Eyes in subjects with Primary Sjögren's Syndrome: double blind randomized control study

1차 쇼그렌 증후군 환자의 건성안에 대한 Hydroxychloroquine의 치료 효과: 이중맹검 연구

2014년 2월

서울대학교 대학원

임상의과학과

윤 창 호

1차 쇼그렌 증후군 환자의 건성안에 대한 Hydroxychloroquine의 치료 효과: 이중맹검 연구

Effect of Hydroxychloroquine Treatment on Dry Eyes in subjects with Primary Sjögren's Syndrome: double blind randomized control study

February 2014

Department of Clinical Medical Sciences

Seoul National University

College of Medicine

Chang Ho Yoon

Effect of Hydroxychloroquine Treatment on Dry Eyes in subjects with Primary Sjögren's Syndrome: double blind randomized control study

지도 교수 김 미 금

이 논문을 의학석사 학위논문으로 제출함

2013년 10월

서울대학교 대학원

임상의과학과

윤 창 호 **(Chang Ho Yoon)**

윤창호의 의학석사 학위논문을 인준함

2014년 1월

위 원 장 위 원 량 (인)

부위원장 김 미 금 (인)

위 원 허 장 원 (인)

Abstract

Introduction: Effect of hydroxychloroquine (HCQ) on dry eye has not been fully determined yet. This study was aimed to compare the 12-weeks efficacy of HCQ medication with placebo in the management of dry eye in Primary Sjögren's Syndrome (pSS).

Methods: A double-blind, randomised control study was conducted in 39 subjects of 153 pSS subjects from May 2011 through August 2013. The diagnosis of Sjögren's syndrome was made on the basis of the classification criteria of the American-European Consensus Group. Subjects received 300 mg HCQ or placebo once daily for 12 weeks. Subjects were evaluated at baseline, 6 weeks and 12 weeks and re-visited at 16 weeks after drug discontinuance. Fluorescein staining score, Schirmer test score, tear film break-up time (TBUT), and ocular surface disease index (OSDI) were measured, and tears and blood were collected for ESR, IL-6, IL-17, B-cell activating factor (BAFF) and Th17 cell analysis. Color test and fundus were examined to monitor HCQ complications.

Results: Twenty-six subjects completed follow-ups. Fluorescein staining score and Schirmer test score did not differ significantly. The OSDI improved

with medication, but this was not insignificant between the groups. TBUT, Serum IL-6, ESR, serum and tear BAFF, and the proportion of Th17 cells did not change in either group.

Conclusions: HCQ at 300 mg daily has no apparent clinical benefit on dry eye and systemic inflammation in pSS.

Keywords: Hydroxychloroquine, Sjögren's Syndrome, Double blind randomized prospective study, OSDI

Student Number: 2011-22002

TABLE OF CONTENTS

LISTS of TABLE and FIGURES

Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease involving lacrimal and salivary glands with resultant keratoconjunctivitis sicca and xerostomia, and lymphocytic infiltration of exocrine glands and epithelium is a common pathological finding [1]. It is one of the most prevalent rheumatologic diseases and has female predominance [2]. About one-third of SS patients have extraglandular systemic involvement, and SS patients with or without other autoimmune rheumatic disease are defined as secondary SS (sSS) or primary SS (pSS), respectively. In 2002, the American–European Consensus Group defined the rules for classifying between pSS and sSS mainly by using a serologic marker (anti-Ro/La antibody level) and histopathology of salivary glands [3].

Regarding dry eye in SS, conjunctival and corneal staining tests, the Schirmer test, and symptoms are worse in the patients with than without SS among aqueous-deficient dry eye patients [4]. Conjunctivocorneal epithelial disintegration results in blurred vision, severe discomfort, and increased risk of infection. Therefore, severe dry eye in SS patients results in an ongoing poor quality of life.

The first treatment option of dry eye symptoms is topical drugs such as artificial tears or cyclosporine A [2, 5]. However, topical treatment is not usually sufficient for severe dry eye in pSS patients. Because inflammatory markers are increased in SS patients, most systemic medications are aimed at

immunologic pathways [6]. Antimalarial drugs are frequently prescribed to SS patients as non-specific blockers of toll-like receptor 9/7 (TLR9/TLR7), but their effectiveness in dry eye is controversial. Improvement in immunologic markers such as IL-6, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), and B-cell activating factor (BAFF), as well as sicca signs such as the tear break-up test (TBUT) and Schirmer test, has been reported in a few studies after oral management with hydroxychloroquine (HCQ) [7, 8]. However, there is only one two-year double-blind crossover trial for pSS in 19 patients, which demonstrated that HCQ treatment does not have clinical benefits for pSS patients [9].

Therefore, to confirm the effect of oral HCQ treatment in dry eye in pSS, we conducted a prospective randomised, double-blind trial comparing HCQ treatment with a placebo control.

Patients and methods

This randomised controlled trial was conducted at the Department of Ophthalmology at Seoul National University Hospital from May 2011 through August 2013, and was registered with ClinicalTrials.gov (Identifier: NCT01601028). Written informed consent was obtained from all subjects, and the study was granted ethical approval by the institutional review board of Seoul National University Hospital (IRB number: H-1104-083-359). This study was conducted in accordance with the tenets of the Declaration of Helsinki.

Patients

Inclusion criteria were (1) adult (aged >18 years) patients with pSS diagnosed on the basis of the 2002 American–European Consensus Group (AECG) criteria [3] and (2) subjects who had the ability to give informed, dated, and signed consent before the beginning of any proceedings related to the trial.

Exclusion criteria were (1) previous treatment with HCQ with insufficient discontinuation time (3 weeks) after discontinuance of treatment; (2) known cardiac disease, respiratory disease, renal disease, or gastrointestinal disease (except gastrooesophageal reflux disease); (3) diabetes mellitus; (4) psoriasis; (5) known drug allergy or hypersensitivity; (6) previous or ongoing treatment

with any other drugs (including topical drugs) that might affect the lacrimal system with insufficient washout time after discontinuance of treatment (e.g. SSRI, anti-histamines, and pilocarpine); (7) closed-angle glaucoma; (8) previous intraocular surgery; (9) macular disease; (10) previous or ongoing treatment with drugs that might have an effect on the macula; (11) pregnancy; and (12) planning pregnancy.

A double-blind, randomised control study was conducted in 39 subjects of 153 pSS subjects for 2 years. We were unable to recruit the intended 60 subjects within the 2-year recruitment period because many subjects with pSS did not meet our inclusion criteria. Most of the subjects did not wish to discontinue previous oral drugs because of severe systemic symptoms. During the study period, 153 pSS patients visited our clinic. Among them, only 67 subjects met all eligibility criteria. Twenty-eight subjects declined to participate in the trial, and 39 subjects were finally enrolled in this study (Figure 1).

During the study period of 2 years, a new criterion of SS was reported [10]. Because this study was designed before the recent criterion was published, we enrolled subjects according to the previous criteria of the American–European Consensus Group to maintain consistency of the subject population.

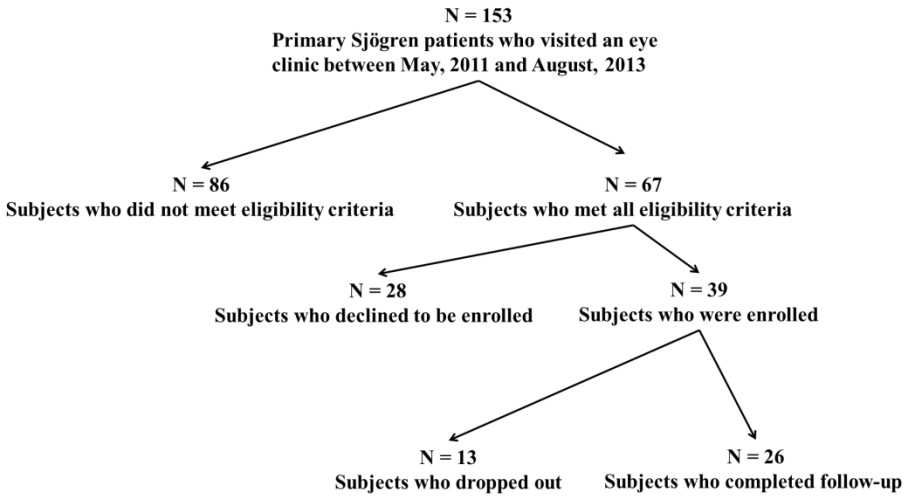

Figure 1. A double-blind randomized control study was conducted in 39 subjects of 153 pSS subjects who visited an eye clinic from May 2011 through August 2013.

Treatment protocol

All subjects underwent initial medical and ophthalmologic history taking and physical examination at the baseline visit (week 0). The study eye was selected as the one which showed the greater corneal fluorescein staining score (on a scale of 0–15; National Eye Institute scale [11] at the baseline visit (Figure 2). Subjects were randomly assigned to the HCQ or placebo group by using sealed randomisation envelopes. The placebo was manufactured identical in appearance to the active drug, and all tablets were kindly supplied by Kyung Poong Pharma Co. Both investigators and subjects were blinded to the treatment assignments. Subjects were instructed to take two tablets (one 200 mg tablet and one 100 mg tablet) of the study medication, giving a total of 300 mg, once daily (qd) for 12 weeks and to report missed doses and adverse events. All subjects used Hyalein Mini ophthalmic solution 0.1% (hyaluronic acid 0.1%; Santen, Osaka, Japan) six times per day. Other topical medications were restricted. Participants returned to the study site at 6 and 12 weeks for efficacy and safety evaluations. After 12 weeks, oral HCQ and placebo medication were withheld, while topical medication was continued in all subjects for 4 weeks. Subjects revisited the clinic at 16 weeks. Schematic schedule arrangement was shown in Figure 3. At each visit, visual acuity, corneal fluorescein staining score using NEI grading, Schirmer test without anaesthesia, tear film break-up time (TBUT), Hardy Rand and Rittler (HRR) colour vision test, and fundus examination were performed. Subjective assessment using the ocular surface disease index questionnaire (OSDI) [12]

was conducted. The OSDI questionnaire was translated to Korean versions from the original English. Tear and blood samples were collected for measurement of IL-6, BAFF, ESR, IL-17, and Th17 cells which secrete IL-17.

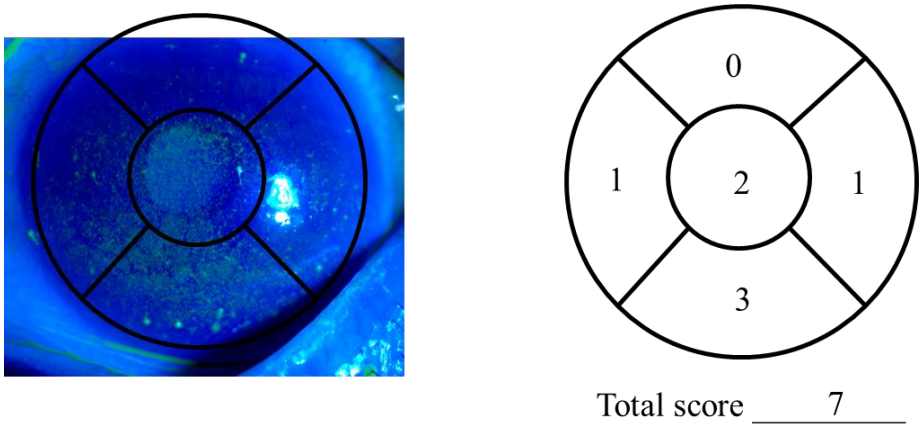

Figure 2. Corneal fluorescein staining score using the National Eye Institute scale (on a scale of 0–15) in a primary Sjögren's syndrome subject

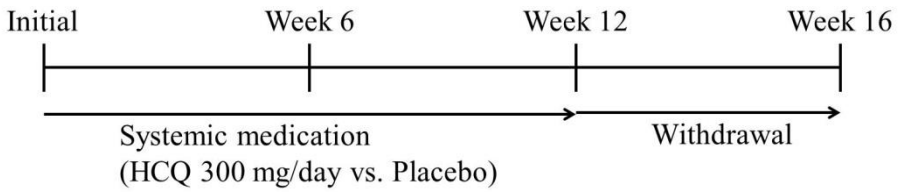

Figure 3. Schematic design of the study protocol regarding time-dependent treatment and examinations

Tear sample collection

Tear fluid was obtained from patients at the same time at baseline and 6, 12, and 16 weeks Tears were collected from the medial and lateral canthus. Topical anaesthesia was not used. To minimise ocular surface irritation, we obtained tear samples by using a Merocel sponge (PVA 0525; Oasis, Glendora, CA, USA) [13]. After collection, the sponge was inserted into a 0.5-mL tube (Eppendorf, Fremont, CA, USA) and the tear fluid was subsequently recovered by centrifugation at 10,000 rpm for 10 min.

Measurement of tear and serum cytokine profiles

Serum ESR was measured by conventional methods in our hospital laboratory on the day of the patient visit. For BAFF and IL-6, 0.5 mL plasma was separated from heparinised peripheral blood and stored together with the tear sample at -70℃ until further examination.

Concentrations of BAFF, IL-6, and IL-17A were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. ELISA using serum was performed in duplicate to ensure the reproducibility of the data. However, ELISA using tears was performed once because of the lack of significant amounts of tears. Tear samples were diluted 1:12.5 and 1:100 for BAFF and IL-17, respectively.

The concentration ranges used for the standard curve were 62.5–4000 pg/mL,

9.38–600 pg/mL, and 15.6–1,000 pg/mL of human recombinant BAFF, IL-6, and IL-17, respectively (R&D Systems).

Flow cytometric analysis for Th17 cells

Flow cytometry was also performed on the same day as the patient visit. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation (BIOCHROM Inc., Cambridge, UK). PBMC were stimulated for 6 h with 50 ng/mL 1-phorbol-12-myristate-13-acetate (PMA; Sigma-A Aldrich, St. Louis, MO, USA) and 1 μg/mL ionomycin (Sigma-A aldrich) in the presence of brefeldin A (BD Bioscience, San Jose, CA, USA) for the final 4 h. The cells were fixed and permeabilised using a BD Cytofix/Cytoperm™ kit (BD Bioscience). The fixed cells were stained with anti-CD3-APC-Cy7 and anti-CD4-FITC for 30 min at 4℃ for initial surface staining. For intracellular staining, they were incubated with anti-IFN-γ-PE-Cy7 (all from BD Bioscience) and anti-IL-17A-PE (eBioscience, SanDiego, CA) mAb for 60 min at 4℃. Flow cytometric analysis was performed using a FACS BD LSR II (BD Bioscience), and the data were analysed by FlowJo (Treestar, Ashland, OR, USA). Isotype mouse Ig G1-PE-Cy7 (eBioscience) and mouse Ig G1- PE (eBioscience) were used for control.

After four-colour compensation, lymphocytes were gated in the forward scatter (FSC)/side scatter (SSC) gate, and CD4 T cells were then gated by

$CD3^{hi}$ and $CD4^{hi}$ expression; finally, the cells were gated by IFNγ and IL17 (Figure 4). The percentage of Th17 cells was calculated by summating $IL17^{hi}$ cells and IFNγ/IL17 $^{double\ hi}$ cells.

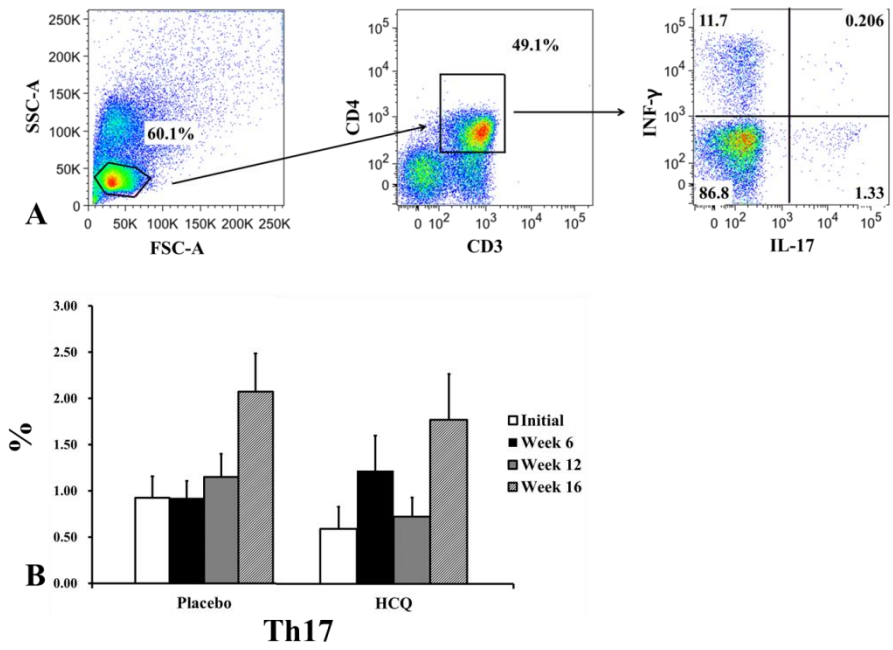

Figure 4. Flow cytometric analysis of Th17 cells which were gated by $CD3^{hi}/CD4^{hi}$ lymphocytes and then gated by IFNγ/IL-17 (A), and time-dependent percent changes of the Th17 cells between the groups (B). Bars represent the mean + standard error.

Sample size calculations

A previous literature review on SS did not include results of tear IL-17 concentration after HCQ medication. Tear IL-17 concentration of SS patients was 504.91 pg/mL and 352.45 pg/mL in the total and mild keratoconjunctivitis sicca groups, respectively, according to this previous report [14]. We assumed the tear IL-17 concentration after HCQ medication would be similar to that of the mild keratoconjunctivitis sicca group. A sample size of 60 patients (30 in each group) was needed to detect 504.91 ± 136.38 pg/mL versus 352.45 ± 136.38 pg/mL between the 2 groups using a 2-sided test with a power of 80% with the significance level controlled at 5%. Sample size calculation was assisted by the Medical Research Collaborating Center of Seoul National University Hospital.

Statistical analysis

Primary analyses of data were based on the per-protocol population, which included all participants who took the 12-week study medication. Statistical analyses were performed using PASW software for Windows (v. 19.0; SPSS Inc., Chicago, IL, USA). A p value of <0.05 was considered statistically significant. The Shapiro–Wilk test was applied to evaluate the data normality. Two groups were compared with a Student's *t*-test (two-tailed) and a Mann–Whitney *U* test according to the normality test result. Differences between the HCQ and placebo groups during the course of the treatment were analysed by

repeated-measures ANOVA and generalized estimating equation when there were non-normality or missing data.

Results

General complications of the subjects and characteristics of drop-out subjects

Of the 39 recruited subjects, 26 were treated per protocol. Thirteen subjects including five in the placebo group and nine in the HCQ group did not complete the study. In the placebo group, three subjects did not keep their follow-up appointments and were unavailable to be reached, one subject suffered dyspepsia, and one subject was found to be unsatisfactory for enrolment. In the HCQ group, two subjects did not keep their follow-up appointments and were unable to be contacted, three subjects suffered dyspepsia, one subject did not have the time for follow-up examinations, two subjects were found deficient in enrolment criteria, and one subject developed incidental subretinal haemorrhage due to occult myopic choroidal neovascularisation (CNV) with accompanying impaired vision 3 days after medication, which was not regarded as a drug-related complication. There were no other drug-related side effects regarding ocular complications (optic neuropathy or maculopathy) or general subjective complications except dyspepsia (10.3%).

Characteristics of the study population at baseline

Of the 26 pSS subjects analysed, 11 subjects were randomised to

receive HCQ and the remaining 15 subjects were randomised to receive placebo. All subjects were women with a mean age of 56.85 years (range, 35–74). The mean (range) age was 55.0 (35–73) years in the placebo group and 59.4 (44–74) years in the HCQ group, which was not significantly different ($p = 0.263$, independent *t*-test). Initial results of serum ESR, IL-6, BAFF, Th17, tear BAFF, TBUT, Schirmer score, corneal staining score, and OSDI are shown in Table 1. There were no significant differences between the groups in all of the demographic factors and parameters.

Changes in the parameters

Changes in parameters are shown in Table 2 and Figures 4 and 5. Regarding systemic inflammatory parameters, mean ESR tended to continuously increase in the placebo group and decrease in the HCQ group, although there were no significant differences between the groups. Mean serum IL-6, BAFF, proportion of Th17 cells, tear BAFF and BUT did not show statistically significant differences between the groups. Tear IL-17 did not detect almost of the samples.

Table 1. Demographics and initial characteristics of the subjects

	Total (N = 26)	Placebo (n = 15)	HCQ (n = 11)	P
Age (yr)	56.85 ± 9.66	55.0 ± 9.72	59.4 ± 9.42	0.263*
Serum ESR (mm/h)	23.42 ± 14.78	19.67 ± 12.08	28.55 ± 17.08	0.133*
Serum IL-6 (pg/ml)	4.31 ± 7.45	4.20 ± 4.90	4.45 ± 10.25	0.134†
Th17 (%)	0.80 ± 0.74	0.93 ± 0.80	0.61 ± 0.70	0.340†
Serum BAFF (pg/ml)	2300 ± 859	2327 ± 1028	2261 ± 601	0.878†
Tear BAFF (pg/ml)	384 ± 220	436 ± 328	417 ± 184	1.000†
TBUT (sec)	2.52 ± 0.85	2.23 ± 0.63	2.78 ± 1.09	0.439†
Schirmer (mm)	4.12 ± 3.90	4.00 ± 3.07	4.27 ± 4.98	0.878†
Corneal Staining Score	3.27 ± 1.71	3.33 ± 1.54	3.18 ± 1.99	0.829*
OSDI	47.92 ± 28.30	43.40 ± 27.33	54.08 ± 29.74	0.352*

*Independent t test, †Mann–Whitney U test

Abbreviations: HCQ, hydroxychloroquine; IL, Interleukin; Th17, T helper 17 cells; BAFF, B-cell activating factor; TBUT, tear film break-up time; OSDI, Ocular Surface Disease Index

Table 2. Changes of the parameters between the HCQ and placebo groups during follow-up

			Baseline	Week 6	Week 12	Week 16	P (baseline - week 12)	P (week 12 vs. 16)
Parameter	ESR (mm/hr)	Placebo	19.67 ± 12.08	20.13 ± 8.12	21.40 ± 11.38	20.20 ± 12.52	0.571*	0.620*
		HCQ	28.55 ± 17.08	27.36 ± 18.49	24.91 ± 20.94	21.09 ± 12.09		
	IL-6 (pg/ml)	Placebo	4.20 ± 4.90	5.31 ± 8.35	5.01 ± 6.83	5.83 ± 8.37	0.451†	0.991*
		HCQ	4.45 ± 10.25	4.77 ± 9.21	5.37 ± 10.32	6.34 ± 14.14		
	Th17 (%)	Placebo	0.93 ± 0.80	1.05 ± 0.73	1.14 ± 0.98	2.07 ± 1.48	0.199†	0.566*
		HCQ	0.61 ± 0.70	1.05 ± 1.32	0.91 ± 0.77	1.79 ± 1.65		
	Serum BAFF (pg/ml)	Placebo	2327 ± 1028	2513 ± 1903	2480 ± 1410	N/A	0.340†	N/A
		HCQ	2261 ± 601	2071 ± 492	2116 ± 711			
	Tear BAFF (pg/ml)	Placebo	436 ± 328	141 ± 56	427 ± 256	N/A	0.723†	N/A
		HCQ	417 ± 184	1094 ± 1418	1113 ± 1466			
	TBUT (sec)	Placebo	2.23 ± 0.63	2.60 ± 0.83	2.87 ± 0.99	2.57 ± 0.76	0.125†	0.746*
		HCQ	2.78 ± 1.09	2.60 ± 0.84	2.45 ± 0.52	2.18 ± 0.40		
	Schirmer (mm)	Placebo	4.00 ± 3.07	4.33 ± 4.22	3.20 ± 2.68	3.43 ± 2.14	0.136†	0.958*
		HCQ	4.27 ± 4.98	2.50 ± 1.08	2.82 ± 2.40	2.91 ± 2.26		
	Corneal Staining Score	Placebo	3.33 ± 1.54	3.20 ± 2.01	3.67 ± 1.54	4.07 ± 2.09	0.128†	0.524*
		HCQ	3.18 ± 1.99	3.10 ± 2.18	2.54 ± 2.16	2.54 ± 2.42		
	OSDI	Placebo	43.40 ± 27.33	32.45 ± 19.21	30.47 ± 23.47	30.92 ± 28.17	0.209*	0.292*
		HCQ	54.08 ± 29.74	30.37 ± 28.63	22.88 ± 21.51	27.75 ± 21.73		

*Repeated-measures ANOVA, †Linear mixed model

Abbreviations: HCQ, hydroxychloroquine; IL, Interleukin; Th17, T helper 17 cells; BAFF, B-cell activating factor; TBUT, tear film break-up time; OSDI, Ocular Surface Disease Index

With respect to the ocular signs and symptoms, TBUT did not change significantly from baseline to week 12 in either group. The Schirmer test score showed no statistical changes in either group. In corneal staining score, the HCQ group tended to show continuous reduction of the score, although it was not significant. The OSDI was significantly decreased between baseline and weeks 6 and 12 (baseline vs. 6 weeks, $p = 0.024$; baseline vs. 12 weeks, $p = 0.02$; repeated-measures ANOVA with Bonferroni adjustment), suggesting improvement of subjective symptoms. However, there was no statistically significant difference between the HCQ and placebo groups ($p = 0.209$, repeated-measures ANOVA).

After discontinuation of the oral medication, only the proportion of Th17 cells was significantly increased in both groups ($p < 0.01$, repeated-measures ANOVA). However, there was no difference between the groups ($p = 0.566$). Other parameters did not show significant changes after oral medications were discontinued (Table 2).

Discussion

No definite beneficial effect of the use of HCQ in the treatment of dry eye in pSS was found in this study, although there was evidence of improved subjective ocular symptoms. Study results failed to show that HCQ treatment might affect tear production and inflammatory parameters such as ESR, IL-6, BAFF, and Th17 cells in pSS patients

The main anti-inflammatory mechanism of HCQ is considered non-specific antagonism at TLR9 and TLR7 [15, 16]. Circulating DNA- and RNA-containing immune complexes in the blood may stimulate plasmacytoid dendritic cells (pDCs) through TLR9 and TLR7 [16, 17]. Activated pDCs produce IL-6, which can induce co-stimulatory molecules, and stimulated co-stimulatory molecules combined with T cell receptor activate T helper (Th) cells [18, 19]. Recent study has revealed that Th17 cells are up-regulated and involved in the main pathogenic pathways in RA, SS, SLE, and GVHD [20, 21] and many studies have shown that IL-17 level is increased in serum, saliva, and tears in SS patient [14, 21, 22]. In addition, activated pDCs interact with B cells, which produce BAFF. BAFF is an essential homeostatic cytokine for B cells that regulate both innate and adaptive immunity [23]. IL-6 and BAFF are over-expressed in SS patients [24]. The lack of tear production in the SS subjects limited our selection of inflammatory

parameters. Therefore, we analysed IL-6, Th17 cells, BAFF, and ESR in serum and BAFF and IL17 in tears.

We evaluated the effect of HCQ on dry eye as well as systemic changes of inflammatory parameters. Serum and tear BAFF were also not significantly changed. There are two previous reports about BAFF after HCQ treatment: serum BAFF was decreased after HCQ medication; tear BAFF level increased after HCQ discontinuation [8, 25]. But patients of these two reports were constantly took oral HCQ mediation for more than 2 years before enrollment. This could make bias that only patients who was better after HCQ medication were remain at the time of enrollment. Serum ESR, and IL-6 were not significantly changed, which was comparable with previous study [9]. However, Tishler et al. [7] reported that serum ESR and IL-6 were significantly decreased after 12 months, but not during 6 months, of HCQ treatment (200 mg daily). Possible reasons for the lack of changes in ESR and IL-6 are as follows: (1) HCQ might have no clinically beneficial anti-inflammatory effect. (2) Short-term usage (12 weeks) might have been inadequate to stop the chronic inflammatory process and vicious cycle although effective drug concentration is reached in the serum within 2 weeks. (3) Previous studies of HCQ in SS patients permitted low dose of oral steroid or topical cyclosporine A [8, 26]. Our

patients were not allowed any other oral or topical anti-inflammatory medication, which might have caused the results in our study to differ from those of the other studies. (4) The small number of enrolled subjects might have affected statistical significance. Disappointingly, HCQ showed no definite effect on Th17 cells. Possible reasons are as mentioned above. In addition, the dosage of 300 mg daily might not be sufficient to downgrade aggravated inflammation such as activated Th17 cells.

Regarding ocular changes, the Schirmer test score and BUT did not change significantly during follow-up after HCQ treatment and showed no difference between the groups. This suggests that HCQ treatment did not affect the tear production in this study. The presumed reasons for this finding are as follows: (1) HCQ actually has no clinically beneficial anti-inflammatory effect. (2) Considering the age of the subjects (mean age, 56.8 years), most of the lacrimal gland might have been destroyed by chronic inflammation before treatment. HCQ treatment might affect tear production in younger study subjects with early inflammation. HCQ treatment showed time-dependent significant improvement of subjective ocular symptoms in the HCQ group, although there were no significant differences between the HCQ and placebo groups.

Subjective symptoms represented by OSDI were improved while taking the medication, but this was insignificant between the groups. The improved effect may be because of the topical artificial tear or placebo effect. Moreover, the small group numbers might explain why there was no difference between the two groups.

Taken together, HCQ medication did not show significant difference in pSS during the study period. But we did not investigate all of the anti-inflammatory cytokines and inflammatory cells because of the small quantities of collected tears and blood. We might have missed some other anti-inflammatory function of HCQ.

There were several limitations in this study. First, appropriate sample size of the subjects to draw statistical significance could not be achieved. Many of the pSS patients were already taking oral medications such as HCQ, pilocarpine, steroid, or other immune suppressants and were reluctant to stop the medicine for a sufficient wash-out period. Further, budget constraints prevented prolonging the study to enrol the targeted sample number. Second, the study schedule was short. Previous studies which reported significant positive results of HCQ in pSS ran for 12 months, [7, 9] or included cessation of 3 months after 48 months or more of treatment [8]. Third, the small number of enrolled patients might have affected significant differences.

Therefore, further large-scale prospective study is warranted. Nevertheless, our study is worthy of notice because it supports other reports and builds up evidence that HCQ does not have a definite beneficial effect on dry eye or systemic inflammation.

In conclusion, HCQ treatment at a dose of 300 mg daily does not appear to have a clinical benefit on dry eye and systemic inflammation in subjects with pSS.

Acknowledgement

This study was supported by a grant from the Seoul National University Hospital (Project No. 0420110480). Kyung Poong Pharma Co. kindly provided tablets of hydroxychloroquine and placebo.

References

1. Wu CH, Hsieh SC, Lee KL, et al. Pilocarpine hydrochloride for the treatment of xerostomia in patients with Sjogren's syndrome in Taiwan--a double-blind, placebo-controlled trial. J Formos Med Assoc 2006;105(10):796-803.

2. Ramos-Casals M, Tzioufas AG, Stone JH, et al. Treatment of primary Sjogren syndrome: a systematic review. JAMA 2010;304(4):452-60.

3. Vitali C, Bombardieri S, Jonsson R, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61(6):554-8.

4. Liew MS, Zhang M, Kim E, Akpek EK. Prevalence and predictors of Sjogren's syndrome in a prospective cohort of patients with aqueous-deficient dry eye. Br J Ophthalmol 2012;96(12):1498-503.

5. Sall KN, Cohen SM, Christensen MT, Stein JM. An evaluation of the efficacy of a cyclosporine-based dry eye therapy when used with marketed artificial tears as supportive therapy in dry eye. Eye Contact Lens 2006;32(1):21-6.

6. Becker H, Pavenstaedt H, Willeke P. Emerging treatment strategies and potential therapeutic targets in primary Sjogren's syndrome. Inflamm Allergy Drug Targets 2010;9(1):10-9.

7. Tishler M, Yaron I, Shirazi I, Yaron M. Hydroxychloroquine treatment for primary Sjogren's syndrome: its effect on salivary and serum inflammatory markers. Ann Rheum Dis 1999;58(4):253-6.

8. Yavuz S, Asfuroglu E, Bicakcigil M, Toker E. Hydroxychloroquine improves dry eye symptoms of patients with primary Sjogren's syndrome. Rheumatol Int 2011;31(8):1045-9.

9. Kruize AA, Hene RJ, Kallenberg CG, et al. Hydroxychloroquine treatment for primary Sjogren's syndrome: a two year double blind crossover trial. Ann Rheum Dis 1993;52(5):360-4.

10. Shiboski SC, Shiboski CH, Criswell L, et al. American College of Rheumatology classification criteria for Sjogren's syndrome: a data-driven, expert consensus approach in the Sjogren's International Collaborative Clinical Alliance cohort. Arthritis Care Res (Hoboken) 2012;64(4):475-87.

11. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. CLAO J 1995;21(4):221-32.

12. Schiffman RM, Christianson MD, Jacobsen G, et al. Reliability and validity of the Ocular Surface Disease Index. Arch Ophthalmol 2000;118(5):615-21.

13. Acera A, Rocha G, Vecino E, et al. Inflammatory markers in the tears of patients with ocular surface disease. Ophthalmic Res 2008;40(6):315-21.

14. Chung JK, Kim MK, Wee WR. Prognostic factors for the clinical severity of keratoconjunctivitis sicca in patients with Sjogren's syndrome. Br J Ophthalmol 2012;96(2):240-5.

15. da Silva JC, Mariz HA, da Rocha LF, Jr., et al. Hydroxychloroquine decreases Th17-related cytokines in systemic lupus erythematosus and rheumatoid arthritis patients. Clinics (Sao Paulo) 2013;68(6):766-71.

16. Kuznik A, Bencina M, Svajger U, et al. Mechanism of endosomal TLR

inhibition by antimalarial drugs and imidazoquinolines. J Immunol 2011;186(8):4794-804.

17. Seillet C, Laffont S, Tremollieres F, et al. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor alpha signaling. Blood 2012;119(2):454-64.

18. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Am J Pathol 2012;181(1):8-18.

19. Mills KH. TLR9 turns the tide on Treg cells. Immunity 2008;29(4):518-20.

20. Katsifis GE, Rekka S, Moutsopoulos NM, et al. Systemic and local interleukin-17 and linked cytokines associated with Sjogren's syndrome immunopathogenesis. Am J Pathol 2009;175(3):1167-77.

21. Oh JY, Kim MK, Choi HJ, et al. Investigating the relationship between serum interleukin-17 levels and systemic immune-mediated disease in patients with dry eye syndrome. Korean J Ophthalmol 2011;25(2):73-6.

22. Nguyen CQ, Hu MH, Li Y, et al. Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjogren's syndrome: findings in humans and mice. Arthritis Rheum 2008;58(3):734-43.

23. Moisini I, Davidson A. BAFF: a local and systemic target in autoimmune diseases. Clin Exp Immunol 2009;158(2):155-63.

24. Roescher N, Tak PP, Illei GG. Cytokines in Sjogren's syndrome: potential therapeutic targets. Ann Rheum Dis 2010;69(6):945-8.

25. Mumcu G, Bicakcigil M, Yilmaz N, et al. Salivary and Serum B-cell

Activating Factor (BAFF) Levels after Hydroxychloroquine Treatment in Primary Sjogren's Syndrome. Oral Health Prev Dent 2013;11(3):229-34.

26. Fox RI, Dixon R, Guarrasi V, Krubel S. Treatment of primary Sjogren's syndrome with hydroxychloroquine: a retrospective, open-label study. Lupus 1996;5 Suppl 1:S31-6.

국 문 초 록

서론: Hydroxychloroquine (HCQ)은 쇼그렌 증후군 환자에게 오래 전부터 사용되어 온 약으로 아직 기전에 대한 것은 잘 밝혀져 있지 않다. 본 연구는 1차 쇼그렌 환자에서 12주간의 HCQ의 복용 효과를 전향적 무작위 이중 맹검법을 이용한 위약 대조군 연구방법을 이용하여 확인하고자 하였다.

방법: 2011년 5월부터 2013년 8월까지 내원한 153명의 1차 쇼그렌 환자 중 39명을 대상으로 이중맹검 위약-대조군 시험을 시행하였다. 1차 쇼그렌 증후군 환자의 진단은 American-European Consensus Group의 진단기준을 사용하였다. 환자군을 하루 300 mg 의 HCQ 복용군과 위약군으로 나누었다. 환자는 복용 시작 직전, 복용 6주째, 복용 12주째 그리고 복용을 4주간 중단 후 내원하였다. 내원시마다 각막염색검사, 쉬머검사, 눈물막 파괴시간, 안구표면질환지수 (OSDI)를 측정하였고, 혈액의 ESR, IL-6, B-cell activating factor (BAFF), Th17세포, 눈물의 BAFF, IL-17의 검사를 위해 내원시마다 혈액과 눈물을 채취하였다. 한편 망막 독성여부를 보기 위해서 색각검사, 안저검사를 내원시마다 검진하였다.

결과: 26명의 환자가 최종적으로 모집되었다. 각막염색수치 및 쉬머 검사는 두 군간의 차이가 없었다. 반복측정분산분석으로 분석시 안구표면질환지수는 26명의 환자에서 유의하게 호전되었으나 (P = 0.02) 위약군과 차이가 없었다 (p = 0.209). 눈물막 파괴시간, 혈액의 IL-6, ESR, BAFF, Th17 세포의 비율은 차이 없었고 눈물의 BAFF역시 두 군에서 모두 치료기간 동안 차이를 보이지 않았다.

결론: 1차 쇼그렌증후군 환자에서 하루 300 mg의 HCQ복용은 각막건조증과 전신 염증상태의 호전에 이득이 없었다.

주요어: hydroxychloroquine, 쇼그렌 증후군, 전향적 무작위 이중 맹검법, 안구표면질환지수

학　번: 2011 – 22002