한국임상약학회지 제26권 제3호
Korean J Clin Pharm, Vol. 26, No. 3, 2016
Original Article

Korean Journal of Clinical Pharmacy
Official Journal of
Korean College of Clinical Pharmacy
Available online at http://www.kccp.or.kr
pISSN: 1226-6051

간헐적 발열 반응에 의한 세포 손상과 이와 관련된 탈모 치료를 위한 신 후보물질 연구

임성실[1] · 문홍섭[2]*
[1]가톨릭대학교 약학대학, [2]국립목포대학교 약학대학 천연약물연구소
(2016년 6월 22일 접수 · 2016년 8월 31일 수정 · 2016년 9월 6일 승인)

Effects of Early Cell Damage from Repetitive Intermittent Fever Exposure in Alopecia Progression and Evaluation of New Candidate Drugs: Ibuprofen, Menthol, and Cetirizine

Sung Cil Lim[1] and Hong Seop Moon[2]*
[1]College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea
[2]College of Pharmacy and Natural Medicine Research Institute, Mokpo National University, Muan, Jeonnam 58554, Republic of Korea
(Received June 22, 2016 · Revised August 31, 2016 · Accepted September 6, 2016)

ABSTRACT

Background: Alopecia areata (AA) is a very disturbing and expensive disorder in which the exact etiology is not known and it is yet to be treated completely well. Most alopecia patients exhibit some inflammation in the hair follicles regardless of the causes. The clinical symptoms of alopecia present very diversely while the prime symptom is local intermittent fever which are related to inflamed cells. **Methods:** This study aimed to evaluate how repetitive intermittent fever can damage the normal human dermal fibroblast (NHDF) cells and investigated the cytotoxic and proliferative effects after application of new candidate drugs (ibuprofen, menthol, cetirizine) for alopecia in comparison to minoxidil. **Results:** This study demonstrated that ibuprofen, menthol, or/and cetirizine can prevent or slow down the damage of NHDF cells from intermittent fever in early alopecia. Aggressive preventative intervention with those drugs before complete destruction of hair follicle by excessive repetitive fever, is a very important step for alopecia therapy and these drugs are recommended as candidate drugs for alopecia in the future. **Conclusion:** Aggressive preventative intervention with drugs before complete destruction of hair follicles (NHDF cells) by excessive repetitive fever is a very important step for alopecia therapy or progression.

KEY WORDS: Alopecia, cetirizine, intermittent fever, ibuprofen, menthol

Alopecia areata (AA) is a phenomenon of unusual excessive hair loss (more than 100 hairs per day) which results in change of appearance.[1)] These days, appearance is an important issue and alopecia is a very disturbing and expensive disorder for people. However, the exact etiology of alopecia is still not largely known but we only assume the causes as excess stress, hormone imbalance, malnutrition, drug, disease inflammation, immune, genetic, or aging etc., and it may be due the complexity of those causes.[1)] Currently a few drugs, finasteride or dutasteride (5 alpha-reductase inhibitors), alfatradiol (17 alpha-estradol) and minoxidil (arterial vasodilators), are approved for progression of alopecia.[2)] Those drugs are only effective to prevent or decrease the loss of hair and are does not completely treat alopecia. Therefore new drugs for alopecia are in dire need. Although many traditional remedies are available, there is still no cure. In addition, those drugs are not completely effective or equally effective for all alopecia patients either. Why is that? Currently we do not know the answer.

Generally, humans have about 100,000-150,000 hairs on their scalp and hair follicles have a life span of about 15

*Correspondence to: Hong Seop Moon, College of Pharmacy, Mokpo National University 1666 Youngsan-ro, Muan-gun, Jeonnam-do 58554, Republic of Korea
Tel: +82-61-450-2685, Fax: +82-61-450-2689
E-mail: hbsmoon@mokpo.ac.kr

cycles, which are composed of anagen, catagen and telogen.[3] According to these life cycles, hairs turn over, grow, and regress. Once 15 cycles are finished, there are no more hair growth again. Alopecia can be classified as scarring alopecia and nonscarring alopecia. Since hair follicles are not completely destroyed in nonscarring alopecia, the treatment is only limited for this type of alopecia.[1]

Clinically, symptoms of alopecia are very diverse and are related to inflamed cells. Presenting symptoms include local fever, redness, pruritus, edema or local uncomfortable feeling.[4] Most alopecia patients show some inflammation in their hair follicles (i.e. hair burb, suprabulbar region, isthmus, infundibulum) regardless of the causes. Patients with alopecia present with either local or extensive inflammation of their scalps. Inflammation is the physiological phenomenon of body protection and is involved with many immunological mediators such as prostaglandin, substance P, and histamine, that result in the presentation of diverse inflammation symptoms such as fever, pruritus, edema, redness, etc.[5]

Therefore, control of inflammation in the hair follicle will be very important to prevent or cure alopecia. In this study, I aimed to determine the relationship between inflammation-producing intermittent fever on the scalp of patients with alopecia and the possibility of the improving the control of intermittent fever on the scalp of patients with alopecia. Therefore, I would like to evaluate the effects of early cell damage by repetitive intermittent fever exposure in alopecia progression and discover new candidate drugs for the treatment of alopecia. Especially, fever is a main sign for inflammation in the body. Fever can present locally or extensively in the body and inflammation of alopecia comes with local intermittent fever on the scalp. Although intermittent fever may produce alopecia, we currently do not know whether fever phenomenon occurs before or after alopecia. In my opinion, those intermittent repetitive fever on the scalp (regardless of the types of alopecia) may induce regression of hair follicles in any situation, as those inflammatory hair follicles with regression present with excess prostaglandin D2 (PGD2). Currently, it is supported that topical steroid could be cautiously used for the treatment of inflammation for severe alopecia patients However, use of steroids are not chronically recommended they produce various adverse drug reactions.[6]

The purposes of this study are to evaluate symtoms of alopecia, to prevent or alleviate alopecia progression and second, to expand the FDA application of ibuprofen (anti-inflammation), menthol (anti-fever), or cetirizine (anti-histamine) for early alopecia therapy (before complete destroying of hair follicle) by controlling of intermittent fever on scalp of alopecia patients.[6,7]

Scalp fever in alopecia patients occur intermittently and repetitively.[8] The intermittent scalp fever becomes more frequently and repetitive when people are under increased stress. People in the modern society are under more stress. Although intermittent fever can cause inflammation of hair follicles slowly, it does not cause a complete destruction of the hair follicles at once. Patients with intermittent fever on the scalp present with hair thinning and loss of hair until complete destruction of the hair follicles.[9] If factors that affect the timing of hair follicles by intermittent fever could be controlled, alopecia may be prevented or the disease process may slow down.

Therefore, the present study evaluated the effects on the control of intermittent fever by application of drugs, menthol (for controlling of intermittent fever on the scalp), ibuprofen (for decreasing PG synthesis in inflammatory situation caused by intermittent fever), and cetirizine (for antagonizing histamine in inflammatory situation caused by intermittent fever) and compared all the results by evaluating each drug with minoxidil, a drug with FDA indication of alopecia treatment, under the same condition.[4,10,11] Intermittent fever was defined as the intermittent exposure of hair cells under 37-38C temperature for 5 minutes. The study was performed on normal human dermal fibroblast (NHDF) cells to evaluate the cell cytotoxicity and proliferation by MTS assay between normal cells and inflammatory cells from repetitive intermittent fever.

MATERIALS AND METHODS

Reagents

Ibuprofen, menthol, cetirizine and minoxidil were purchased from Sigma-Aldrich (St. Louis, MO). Cell Titer 96 Aqueous One Solution Reagent [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) - 2H-tetrazolium, inner salt (MTS)] kit for the cell proliferation assay was purchased from Promega (Madison, WI).

Cell culture

NHDF cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in fibroblast medium: DMEM/M199 (4:1) supplemented with

15% FBS (GIBCO, Carlsbad, CA), L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) and were maintained at 5% CO2, 37°C in a humidified condition. Each vial of frozen cells was thawed and maintained for about two months.

MTS assay

For the cytotoxicity assay, cells were seeded (1×10^4 cells per well) in 96-well plates and incubated for 24 h, and then treated with different doses of ibuprofen, menthol, cetirizine and minoxidil. After incubation for 24 h, 48 h, and 72 h, 20 μl of Cell Titer 96 Aqueous One MTS Solution were added and then cells were incubated for 1 h at 37°C in a 5% CO_2 incubator. For the cell proliferation assay, cells were seeded (3×10^3 cells per well) in 96-well plates and incubated for 24 h, and then treated with different doses of ibuprofen, menthol, cetirizine and minoxidil. To examine the effects of temperature changes, cells were incubated at 37°C in a 5% CO_2 incubator for 5 min then transferred to 38°C in a 5% CO_2 incubator for 5 min. The procedure was repeated 5, 8, 10 or 15 times. After incubation for 48 h, 20 μl of CellTiter96 Aqueous One MTS Solution were added and then cells were incubated for 1 h at 37°C in a 5% CO_2 incubator. Absorbance was measured at 492 nm by microplate reader (Bio-Rad, Hercules, CA).

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical comparisons between experimental groups were performed with unpaired Student's t-tests. The p-values of less than 0.05 were considered significant.

RESULTS

Cytotoxic effects

In the present study, the cytotoxic effects of ibuprofen, menthol, cetirizine and minoxidil were first examined in normal cell culture conditions. NHDF cells that were treated with DMSO (vehicle), 50, 250, 500 or 1000 μM of ibuprofen and menthol for 0, 24, 48 or 72 h did not exhibit cytotoxicity (Fig. 1A, B). NHDF cells that were treated with DMSO, 12.5, 25, 50, or 100 μM of cetirizine and DMSO, 0.125, 0.25, 0.5 or 1 μM of minoxidil did not affect the cell toxicity (Fig. 1C, D). This indicates that ibuprofen, menthol, cetirizine and minoxidil did not have cytotoxic effects in the NHDF cells. By previous studies of other researchers, all evaluated concentrations were determined.

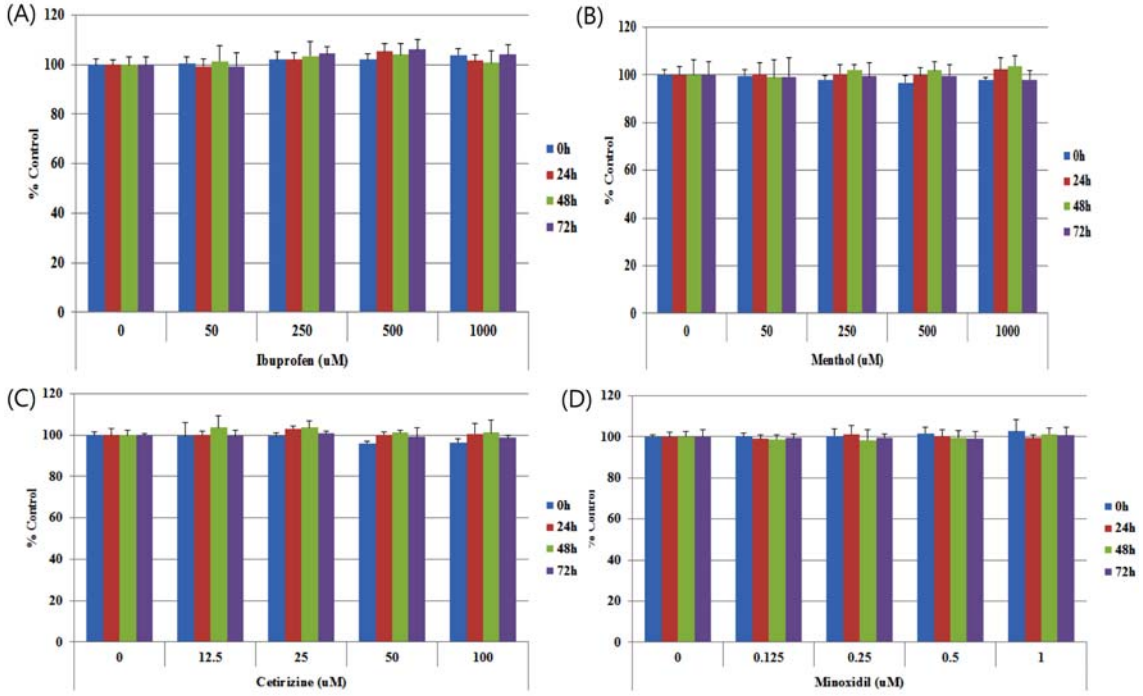

Fig. 1. Cytotoxic effects on human follicle dermal papilla cell by each drug: (A) ibuprofen, (B) menthol, (C) cetirizine, and (D) minoxidil. Cells were treated for 0, 24, 48, 72h with various concentrations of ibuprofen, menthol, cetirizine or minoxidil. Data are represented as means ± SD of values from triplicate samples and similar results were obtained from two independent experiments.

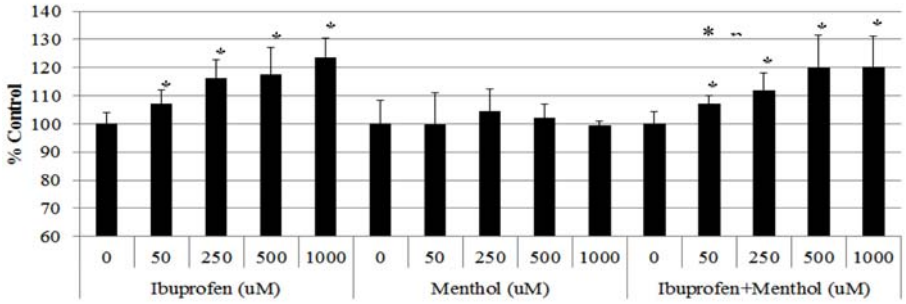

Fig. 2. Proliferative effects of each drug on human follicle dermal papilla cell: ibuprofen, menthol, and ibuprofen with menthol. Cells were treated for 48h with 0, 50, 250, 500 or 1000 μM concentrations of ibuprofen, menthol or ibuprofen plus menthol. The asterisks (*, $p<0.05$) indicate a significant increase in proliferation with ibuprofen or ibuprofen plus menthol treatment compared with untreated control. Data are represented as means ± SD of values from triplicate samples and similar results were obtained from two independent experiments.

Cell proliferative effects

To verify the proliferative effects of ibuprofen and menthol in NHDF cells, the cell growth assay was performed. NHDF cells proliferated by the treatment of ibuprofen in a dose dependent manner (Fig. 2), while menthol, and the treatment with ibuprofen and menthol has not synergic effect on proliferation of NHDF cells (Fig. 2).

Cell proliferative effects post intermittent fever treatments

Next, the effects of ibuprofen, menthol, and minoxidil under fever were investigated. Incubation temperature were changed from 37°C to 38°C at 5 min intervals and this process was repeated 0, 5, 8, 10, and 15 times and then incubated at 37°C for 48 h. As shown in Fig. 3, The proliferation of NHDF cells treated with ibuprofen, menthol or minoxidil were increased when the temperature changes were performed 5 times (Fig. 3B) compared with no temperature change (Fig. 3A). NHDF cells treated with ibuprofen or menthol were affected when the temperature changes were performed 8 and 10 times (Fig. 3C, D), but minoxidil did not affect cell proliferation (Fig. 3C, D). NHDF cells treated with ibuprofen, menthol or minoxidil were not responsive when temperature changes were performed 15 times (Fig. 3E). Furthermore, NHDF cells treated with ibuprofen, menthol, cetirizine or minoxidil were proliferated when the temperature changes were performed 5 times (Fig. 4). Normal cells with no intermittent fever treatment showed no change under each drug application.

DISCUSSION

The main clinical symptom of alopecia is intermittent type of scalp fever.[11] Scalp fever can cause inflammation which induces regression of hair follicle.[12] Scalp fever of alopecia patients occur in an intermittent pattern and the intermittent pattern fever on scalp occur and increases in frequency when people are under the stress.[9] Intermittent fever can cause inflammation of hair follicle slowly not complete destruction at once. That is, hair follicle will be under the inflammatory situation[13] as the repetitive exposure of intermittent fever and will be destructed completely someday. Therefore actively control of intermittent fever is very important intervention in early alopecia situation. The reason is that intermittent fever affects the hair follicle slowly until complete destruction of hair follicle. It showed the similar pattern for hair loss process in alopecia patient. Patients occurring intermittent fever on scalp show hair thinner and loss of hair until complete destruction of hair follicle. If those affecting time of hair follicle by intermittent fever will be controlled, alopecia will be prevented or slowed down either. Therefore I evaluate the possibility of new candidate drugs based on the mechanism of controlling inflammation which is interfering of intermittent fever, inflammatory mediators such as prostaglandins or histamin[6] in this study. I compared all the results with minoxidil which is one of best popular drug for alopecia treatment.

First, a nonsteroidal anti-inflammatory drug (NSAID), ibuprofen, inhibits cyclooxygenase which produce prostaglandins, which are inflammatory mediators. Fever can be controlled by NSAIDs from inhibition of prostaglandins. Scalp fever can cause inflammation which may induce regression of hair follicles. Those inflammatory hair follicles with regression showed excess prostaglandin D2 (PGD2). But steroid is not a recommended drug for chronic use because it produces many

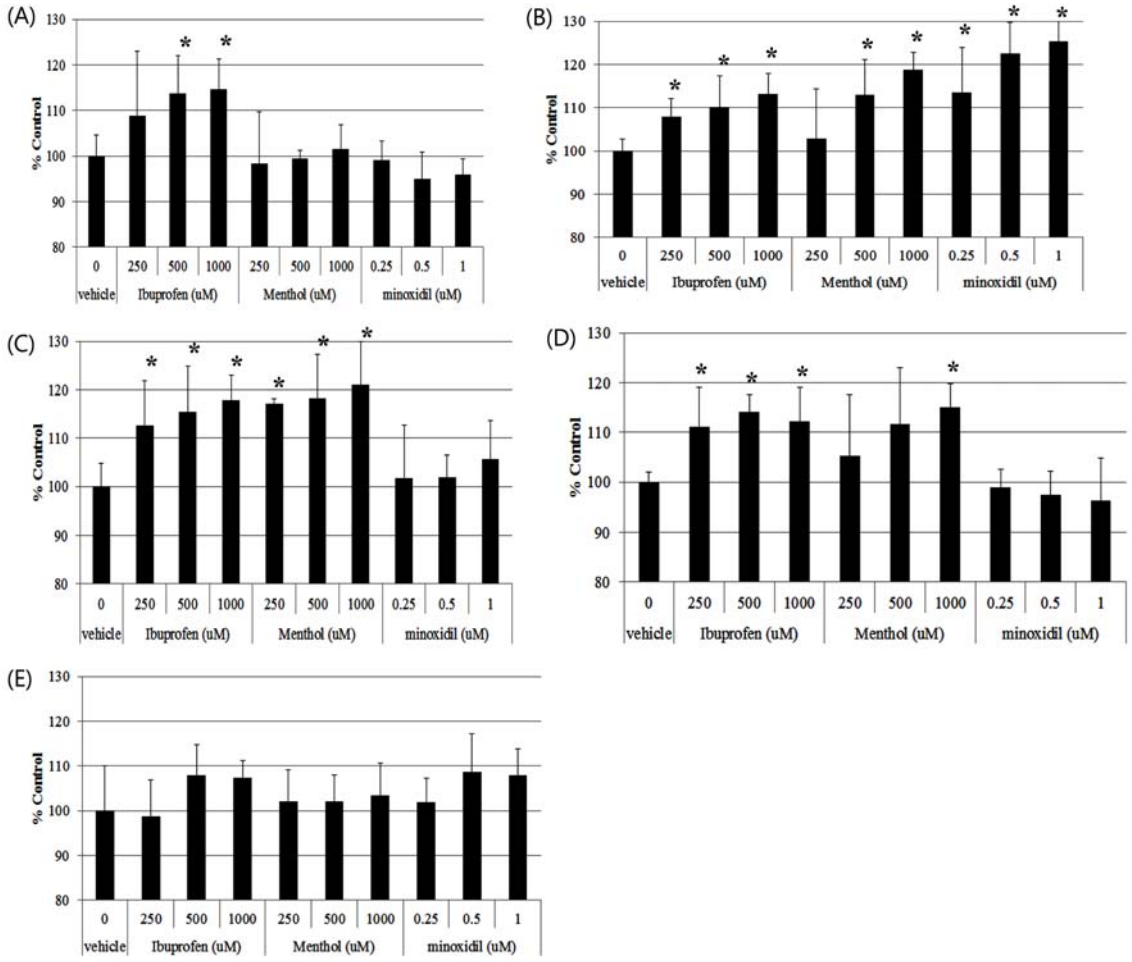

Fig. 3. Proliferative effects of each drug on human follicle dermal papilla Cell after intermittent fever interventions: ibuprofen, menthol, and minoxidil (A: No treatment, B: 5 times-, C: 8 times-, D: 10 times-, E: 15 times) of treatment performed. Cells were treated with various concentrations of ibuprofen, menthol or minoxidil and incubated at 37°C in a 5% CO_2 incubator for 5 min then transferred to 38°C in a 5% CO_2 incubator for 5 min and repeated the temperature changes for 5, 8, 10 or 15 times. Then, incubated for 48h and detected by MTS assay. The asterisks (*, $p<0.05$) indicate a significant increase in proliferation with ibuprofen, menthol or minoxidil treatment compared with untreated control. Data are represented as means ± SD of values from triplicate samples and similar results were obtained from two independent experiments.

adverse reactions.[6)]

Secondly, menthol, a natural product of the peppermint plant Mentha x piperita (Lamiacease) is a monoterpene which is widely used for antipruritic, antiseptic, analgesic, and cooling formulations. Despite of widespread use, the menthol's complete mechanism is not studied.[14)] Menthol acts upon TRPM8 receptors by rapidly increasing intracellular calcium and mobilizing calcium flux through the channels to induce cold response signals at the application site. Menthol exhibits cytotoxicity in cancer cells and induces reduction of malignant cell growth, and engages excitation of GABA receptors and sodium ion channels resulting analgesia, aside from its cold-inducing sensation capabilities.[15)] Only menthol elicits the mechanism which is similarly related to the cooling sensation from low temperature of TRPM (Transient Receptor Potential Melastatin) 8 receptors. Like this, many different target actions showed the possibility[14)] that menthol may be a valuable compound for treatment in the future. Most alopecia patients feel the fever sensation in their scalp, which increases the pruritus, inflammation, weakening and decrease of hair width and numbers, etc.[16)] The fever type from alopecia patients was intermittent, not consistent.

Thirdly, an antihistamine drug, cetirizine showed transient regrowth of sparse vellus hair, which occurred in some severe

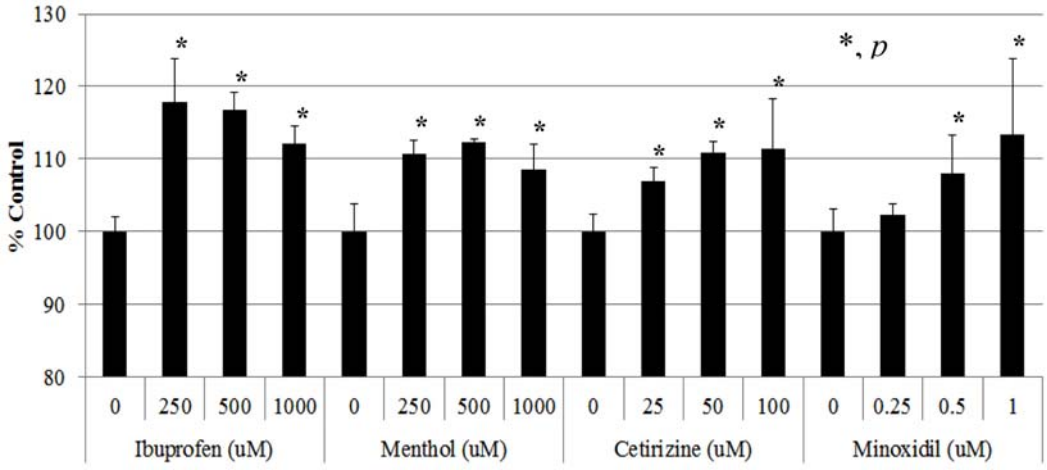

Fig. 4. Proliferative effects of each drug on human follicle dermal papilla Cell: ibuprofen, menthol, cetirizine and minoxidil after five times of intermittent temperature changes performed (optimal condition for drug response). The asterisks (*, $p<0.05$) indicate a significant increase in proliferation with ibuprofen, menthol, cetirizine or minoxidil treatment compared with untreated control. Data are represented as means ± SD of values from triplicate samples.

chronic alopecia patients or atopic alopecia patients. Histamine is one of inflammatory mediators but the exact mechanism for transient regrowth of hair is unknown. Antihistamines may prevent inflammation from histamine release and may improve results in severe alopecia patients.[17,18)]

In this study, the cytotoxic effects of ibuprofen, menthol, cetirizine and minoxidil were first examined in normal NHDF cell culture conditions. All evaluating concentrations of each drug were followed by previous other studies. According to the results, ibuprofen, menthol, cetirizine and minoxidil did not exhibit cytotoxicity in the NHDF cells, which indicates that all evaluating drugs can be use as candidates for alopecia therapy because they lack cytotoxicity (Fig. 1A, 1B, 1C, 1D).

To verify the proliferative effects of ibuprofen and menthol in NHDF cells, the cell growth assay was performed. NHDF cells were proliferated by treatment of ibuprofen in a dose dependent manner. However, menthol did not affect the proliferation of NHDF cells and neither did the synergic treatment of ibuprofen and menthol simultaneously. This indicates that only ibuprofen exhibited the proliferation and it could be a possible candidate drug for alopecia in a topical dosage form. In addition, menthol did not exhibit negative proliferation on NHDF cells and may be used as an additive to ibuprofen for alopecia therapy (Fig. 2).

Next, the effects of ibuprofen, menthol, and minoxidil at the condition of fever conditions were investigated with various numbers of changes in temperature. The incubation temperature was changed 37°C to 38°C, 5 min intervals and the repetition was 0, 5, 8, 10 or 15 times and then, incubated at 37°C for 48 h. Five conditions were set up (3A, 3B, 3C, 3D, and 3E) to evaluate the effect of intermittent fever before application of each drug. As the results shown Fig. 3, The proliferation of NHDF cells treated with ibuprofen, menthol or minoxidil were increased in the 5 times temperature changes (Fig. 3B) compared with the no change of temperature (Fig. 3A). NHDF cells treated with ibuprofen or menthol were affected in the 8 and 10 times temperature (Fig. 3C, D), but minoxidil didn't effect to cell proliferation (Fig. 3C, D). NHDF cells treated with Ibuprofen, menthol or minoxidil were not response in the 15 times temperature changes (Fig. 3E). In Figure 3A, NHDF cells exhibited the proliferation effects only with ibuprofen (at 500 mcg concentration) but not with other drugs. In Figure 3B, all of drugs exhibited proliferation effects, which indicates that this is the optimal condition for each drug's response. In Figure 3C, only ibuprofen and menthol affected the proliferation effect with statistical significance, but not minoxidil. In Figure 3D, menthol in high concentration and ibuprofen (at all concentrations) affected the proliferation effect with statistical significance. In Figure 3E, all drugs showed no effect, which indicates that excessive intermittent fever (15 times) produce severe cell damage and recovery is not possible with treatment. The present study suggests that the optimal condition for drug treatment would be after five times of intermittent fever condition. This suggests that early

aggressive pharmacotherapy for alopecia may be a very important factor in achieving the optimal efficacy of drug therapy. In early NHDF cell damage by intermittent fever, ibuprofen and/or menthol were equally effective as minoxidil and both drugs may be new candidate drugs for alopecia treatment. Therefore, ibuprofen and/or menthol may consider expanding their application from FDA to add indication for treatment of alopecia. After the intermittent fever conditions were applied eight times, minoxidil exhibited no effects, which suggests that minoxidil is more effective in the early stages of alopecia than late stages. Additionally, since menthol has no cytotoxic effect on normal cells with good effects on damaged cells by intermittent fever, menthol can be used as an additive to other drugs, such as ibuprofen or minoxidil, and can be utilized as a combined product.

Furthermore, NHDF cells treated with ibuprofen, menthol, cetirizine or minoxidil proliferated when the temperature changes were performed five times (Fig. 4). This evaluated the effects of histamine, which is one of the inflammatory mediators for damaged NHDF cells from intermittent fever. Therefore cetirizine, an antihistamine, was applied and it was evaluated for proliferation effects. The result showed that proliferation effects of cetirizine were equal to ibuprofen and menthol and it was superior to minoxidil. This suggests that histamine, as inflammatory mediator, is involved in the damage from intermittent fever treatments.

This study aimed to evaluate the effect and the degree of damage by intermittent fever on NHDF cells. That is how intermittent fever can damage NHDF cells and may evaluate those damage is inflammation or not. I evaluated the response of the inflammatory damage by intermittent fever in each drug application (ibuprofen, menthol, cetirizine, minoxidil) and examined the possibility of new drugs for alopecia.[3,5,7] This study is very meaningful in new possibility of expansion for currently used drugs with FDA indication.[19]

In conclusion, this study showed the following results. 1) Exposure of intermittent repetitive fever produced damage to NHDF cells and although it is an intermittent fever, no drugs (including of minoxidil) is effective under excessive exposure. This indicates that aggressive prevention of intermittent fever is very important in decreasing the damage of NHDF cells and preventing the progression of alopecia. 2) Menthol did not exhibit cytotoxicity on normal NHDF cells and no synergy effects were observed with ibuprofen. However, menthol exhibited similar degree of proliferation effects as minoxidil on NHDF cells post exposure of intermittent fever when applied five times, and resulted in better outcomes when intermittent fever were applied eight to ten times. However, there were no proliferation effects when excessive repetitive intermittent fever were applied (15 times), which is similar to the results from other drugs. Since menthol has no cytotoxicity on normal cells and were more effective in early damage cells, menthol may be used as a preventative drug alone or with combination of other effective drugs in the early alopecia. 3) Ibuprofen showed no cytotoxicity and was the only drug with proliferative effect. The proliferative effects from ibuprofen were observed from mild (0 times) to moderate exposure (10 times) of intermittent fever and these proliferative effects was superior to minoxidil. However, ibuprofen, like other drugs, did not show proliferative effects to excessive exposure of intermittent fever (15 times). This suggests that ibuprofen may be applicable as topical dosage form for alopecia and this topical ibuprofen may be used from earlier stages if concentration is appropriate in which adverse drug reactions may be minimized in other areas of the body. 4) In the case of cetirizine, at the optimal condition in which all investigated drugs showed proliferative effects. (5 times exposure), cetirizine showed the proliferative effects like ibuprofen and menthol and these results were better than the results from minoxidil 5) Minoxidil, a drug with KFDA indication for alopecia, showed proliferation effect only when the intermittent fever exposure was performed five times and did not show proliferation effects at no fever exposure (0 times), or repetitive excessive exposure (8 times). This suggests that minoxidil has no proliferation effects on normal cells or on chronically progressive damaged cells. We can predict that minoxidil is effective on the cells only from the beginning to when there is some degree of damage, but not when the damage on the cell is complete.

CONCLUSION

In conclusion, intermittent fever can affect the proliferation of normal NHDF cells and NHDF cells treated with ibuprofen, menthol, and cetirizine showed diverse proliferation respectively. However, in the setting of excessive repetitive intermittent fever, NHDF cells showed no effect from any of the drugs. Therefore, aggressive preventative intervention with drugs before complete destruction of hair follicles (NHDF cells) by excessive repetitive fever is a very important step for alopecia

therapy or progression. Ibuprofen, menthol, and/or cetirizine can prevent or slow down the damage of hair follicles (NHDF cells) from intermittent fever in early alopecia and could be recommended as candidate drugs for alopecia in the future.

ACKNOWLEDGMENTS

This study was supported by the Research Fund, 2016 of The Catholic University of Korea.

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