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RESEARCH ARTICLE

# Dual Wavelength LEDs Induce Reactive Oxygen Species and Nitric Oxide That Inhibit the Production of Dihydrotestosterone by 5-α Reductase

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## ABSTRACT

Androgenetic alopecia (AGA) causes balding in approximately 50% of adults. One primary cause of AGA is synthesis of dihydrotestosterone from testosterone by 5-α reductase. Systemic pharmaceutical interventions have potentially serious side effects, necessitating development of localized interventions. One such approach is administration of red light via low level light therapy (LLLT), which has promising clinical data. However, the LLLT mechanism of action remains unclear. We investigated the ability of LLLT to stimulate nitric oxide (NO) and the role of NO in inhibition of DHT synthesis. Our results show that red and red-orange light induce NO release in a cell-free platform. In A549 and HEK293T cells, we demonstrate 620 and 660 nm LED-emitted light stimulates the production of NO, reactive oxygen species (ROS), and decreases DHT synthesis. These results provide a plausible mechanism of action for LLLT employing LED-emitted red and red-orange wavelengths of light to treat AGA.

## 1 | Introduction

Androgenetic alopecia (AGA) is the leading cause of pattern baldness, afflicting approximately 50% of men and women by the age of 50 [1]. In men, AGA results in scalp hair loss in the vertex and frontal regions, while hair loss presents as diffuse thinning across the scalp in women [1]. AGA results from androgenic effects in individuals with specific genetic factors [1]. Specifically, the potent androgen dihydrotestosterone (DHT), a derivative of testosterone whose synthesis is catalyzed by the enzyme 5-α reductase, has been strongly associated with the development of AGA in genetically susceptible individuals [1].

Multiple treatment modalities exist to treat AGA, including minoxidil, finasteride, dutasteride, hair transplantation, and low-level light therapy [2]. Of these, finasteride and dutasteride have a mechanism of action specifically targeting the synthesis of DHT via the direct inhibition of the 5-α reductase enzyme family [2]. Specifically, finasteride inhibits Type II 5-α reductase, while dutasteride inhibits Types I and II 5-α reductase [1, 2]. Clinically, finasteride has been shown to reduce circulating DHT by approximately 70% [3], while dutasteride can reduce circulating DHT by up to 95% [4]; these reductions have been associated with reduced hair loss. Despite this clinical data, both finasteride and dutasteride have off-target effects, including, but not limited to, sexual dysfunction and increased risk of prostate cancer and are contraindicated for use in women of reproductive potential due to potential birth defects [2]. Thus, the continued development of novel, localized therapeutic approaches to treat AGA remains of paramount importance.

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One promising approach to treat AGA is photobiomodulation (PBM) with visible wavelengths of light, commonly referred to as low-level laser (light) therapy (LLLT), which can be administered with lasers or light emitting diodes (LED). LLLT involves the use of visible light, primarily in the red to infrared wavelengths of approximately 600–1070 nm to exert biological effects [5]. A growing body of clinical evidence has demonstrated the efficacy of LLLT-based approaches to treat AGA [6]. In fact, one meta-analysis concluded that LLLT is the superior nonsurgical, nonhormonal approach to treat AGA [7]. The first LLLT device to treat AGA was approved by the United States Food and Drug Administration in 2007 [8]. As of 2021, over 30 home-use LLLT-based medical devices have been approved by the US FDA as a treatment for AGA and hair loss [8].

Despite the clinical success of LLLT-based medical devices in the treatment of AGA, the mechanism of action has remained largely theoretical. Multiple avenues have been proposed, including increased production of ATP, modulation of reactive oxygen species (ROS) and oxidative stress, decreased inflammation, and modulations to gene expression and protein synthesis [9–12]. While LLLT has been shown to conditionally modulate 5-α reductase [13], there has been little direct evidence that LLLT inhibits DHT synthesis. In this study, we explored one potential mechanism of action for LLLT to treat AGA. Specifically, we explored the ability of red-orange (620—630 nm) and red (660 nm) light, on their own and in combination to induce biological responses and the capacity of these responses to inhibit the synthesis of DHT. We demonstrated that 620, 660, and 620 nm + 660 nm light-emitting diodes (LEDs), but not 660 nm lasers, stimulate the production of reactive oxygen species (ROS) in epithelial cell lines. Additionally, dual LED wavelengths stimulated the production of nitric oxide (NO) in vitro, while single wavelength LEDs and lasers did not. Finally, we showed that dual LED wavelengths inhibited the conversion of testosterone to DHT in immortalized epithelial cell cultures, but not in primary hair follicle dermal papilla cells, likely due to the reduced expression or lack of type II 5-α reductase in cultured hair follicle dermal papilla cells [14, 15]. However, in vivo hair follicle dermal papilla cells express both type I and type II 5-α reductase [14]. These results provide evidence that LLLT treatment of AGA functions through NO/ROS inhibition of type II 5-α reductase synthesis of DHT.

## 2 | Materials and Methods

### 2.1 | NO Inhibition of 5-α Reductase

To measure the conversion of testosterone to dihydrotestosterone, 5-α reductase was isolated from frozen human prostate tissue via differential centrifugation [16]. Isolated enzyme was incubated at 37°C with an excess of testosterone and NADPH and terminated with acetonitrile. Testosterone and DHT were extracted from the media and their concentrations determined via GC–MS. For all experiments, a no light or chemical (negative) control and positive (finasteride) control were used to determine assay validity. *S*-nitrosoglutathione (GSNO) was utilized as the NO donor.

### 2.2 | Light-Induced NO Release From Endogenous Stores

To measure the release of NO from endogenous stores by visible light, proteins were nitrosated/nitrosylated using *S*-nitrosoglutathione. Nitrosated proteins were added to a nitric oxide analyzer (NOA) flask in white foam and aluminum foil. The flask was connected to a GE-Sievers Nitric Oxide Analyzer purged with nitrogen gas. The flask was illuminated with 21.82 mW/cm$^2$ of 630 nm LEDs or 15.20 mW/cm$^2$ of 660 nm LEDs for 20 min. The NOA collected NO concentration in parts-per-billion every second; the background prior to illumination was removed.

### 2.3 | Cell Lines

A549 (#CCL-185) and HEK 293 T (#CRL-3216) were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in high glucose DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FetalCloneII (HyClone) and 1% antibiotic-antimycotic (Gibco) and incubated at 37°C/5% $CO_2$. Primary human hair follicle dermal papilla cells (HFDPC) (#602 K-05a) were obtained from Cell Applications Inc. through Fisher Scientific. HFDPC were maintained according to manufacturer's instructions.

### 2.4 | LED And Laser Illumination Platform

An illumination platform was constructed to simulate the distance between light emitters and the surface of the scalp used by LLLT-based caps for androgenetic alopecia (Figure 1). The illumination platform consisted of an optical tower, sample housing, and sensor base. The sensor base was 3-D printed to house a Thorlabs S142CL Integrating Sphere Photodiode Power Sensor and hold a 35 mm × 10 mm tissue culture-treated dish that acts a sample house directly above the Power Sensor. Two optical towers were constructed so that the face of each tested light emitter type (LED and laser) was held 10 mm above the bottom of the sample housing. Light emitters were soldered 13 mm from each other center-to-center perpendicularly on a Printed Circuit Board Assembly (PCBA) to mimic placement in the REVIAN Red cap or another LLLT-based cap (Figure 1A). LEDs and lasers were extracted from the REVIAN Red cap (nominal dose of 1.24 J/cm$^2$ over 10 min) and another LLLT-based cap (nominal dose of 1.10 J/cm$^2$ over 6 min), respectively. The peak wavelengths for the LEDs were 621 and 656 nm and the peak wavelength for the laser was 652 nm (Figure 1B). Each PCBA was connected to a calibrated external power supply and the drive current settings for each were then set to match optical power output from both LEDs and lasers in their respective caps. LEDs were driven at a 10 mA constant current and lasers were driven at a 55 mA constant current via an external and calibrated DC power supply. Corresponding lenses for LED and laser systems were extracted from the REVIAN Red cap and the laser-based LLLT-based cap, respectively, and cut to fit the interior of optical towers. Lenses were placed directly on the tissue culture plate. Samples were illuminated with LEDs for 10 min (nominal dose = 0.44 J/cm$^2$ at 0.74 mW/cm$^2$) or with lasers for 6 min (nominal dose = 0.29 J/cm$^2$ at 0.818 mW/cm$^2$).



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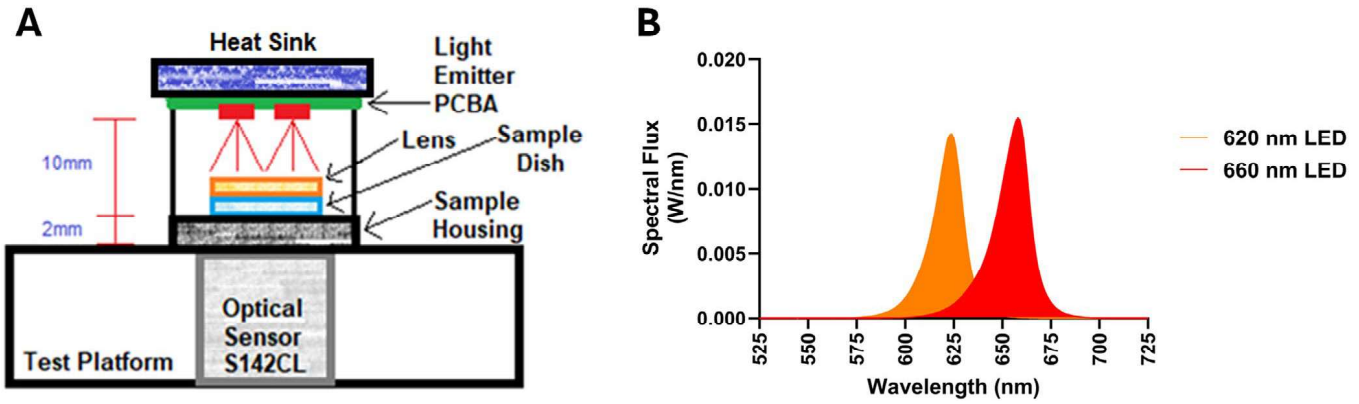


**FIGURE 1** | Translational optics platform. An illumination platform was constructed to simulate the distance between light emitters and the surface of the scalp used by LLLT-based medical devices emitting red and red-orange wavelengths of light as treatment for androgenetic alopecia. (A) The illumination platform consists of an optical tower, sample housing (designed to hold 35 mm × 10 mm tissue culture dish), and sensor base. (B) Independent characterization of the 620 nm and 660 nm LED arrays by measuring the spectral flux. Data are presented as Spectral Flux (Watts/nm).

### 2.5 | ROS Production With CellROX Deep Red Reagent

ROS production was measured with the fluorescent CellROX Deep Red reagent (Invitrogen #C10422) and adapted from manufacturer's instructions. Cells were plated and incubated at 37°C/5% $CO_2$ overnight. Cells were washed twice with PBS. CellROX Deep Red reagent was diluted in PBS. Prior to illumination, diluted CellROX Deep Red reagent was added to cells and incubated at 37°C/5% $CO_2$ until treatment. Following illumination, OptiMEM containing Hoechst stain was added to the cells and then washed with PBS. Cells were visualized within 30 min on the ECHO Revolve fluorescent microscope using the DAPI and Cy5 filters.

### 2.6 | NO Production With DAF-FM Diacetate

NO production was measured with the fluorescent DAF-FM diacetate reagent (Invitrogen #D23844) and adapted from manufacturer's instructions. Cells were plated and incubated at 37°C/5% $CO_2$ overnight. Cells were washed twice with PBS. DAF-FM diacetate was diluted in PBS. Prior to illumination, diluted DAF-FM diacetate reagent was added to cells and incubated at 37°C/5% $CO_2$ until treatment. Following illumination, OptiMEM containing Hoechst stain was added to the cells and then washed with PBS. Cells were incubated at 22°C for 30 min, then visualized on the ECHO Revolve fluorescent microscope using the DAPI and FITC filters.

### 2.7 | DHT Conversion Assay

For the DHT conversion assay, cells were cultured and incubated overnight at 37°C/5% $CO_2$ prior to illumination. Immediately following illumination, 100 nM testosterone-d3 (Sigma-Aldrich #T5536) was added to all cells, except a no testosterone control, which was incubated with only cell culture media. For all experiments, cells without light or chemical treatment (negative) and cells treated with 5 μM finasteride (positive) controls were used to test assay validity. Cell culture supernatants were harvested at 2 h post-illumination (hpi), 6 hpi, and 24 hpi. DHT concentration was measured via DHT competitive ELISA (Abcam #ab283979).

### 2.8 | Statistical Analysis

Statistical analysis was conducted via the Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test. Statistical significance is indicated by $*p \leq 0.05$; $**p \leq 0.005$; $***p \leq 0.0005$;, and $****p < 0.0001$.

## 3 | Results

### 3.1 | NO Inhibits the Conversion of Testosterone to DHT by 5-α Reductase

First, we set out to determine whether nitric oxide (NO) could inhibit the conversion of testosterone to dihydrotestosterone (DHT) by the enzyme 5-α reductase. Using the standard NO donor *S*-nitrosoglutathione (GSNO) and the positive control finasteride, we measured the concentration of DHT following incubation with isolated 5-α reductase enzyme. The remaining DHT concentration was evaluated using GC–MS (Figure 2A).

In comparison to the untreated control, finasteride strongly inhibited the conversion of testosterone to DHT by 5-α reductase in vitro (Figure 2B). At doses as low as 0.1 μM, finasteride reduced the amount of DHT to 10.8% (90.2% inhibition) relative to the untreated group. Interestingly, increasing the finasteride concentration to 1 μM reduced DHT concentrations to 24.8% (75.2% inhibition) relative to the untreated group. However, further increasing the finasteride concentration to 10 μM reduced DHT concentrations to 1.5% (98.5% inhibition) relative to the untreated group. These results indicate that this assay is sufficient to determine inhibition of testosterone conversion to DHT by 5-α reductase in vitro.

The NO donor GSNO displayed a dose-dependent reduction in testosterone conversion to DHT by 5-α reductase (Figure 2B). Dosing with 0.25and 0.50 mM GSNO reduced DHT to 87.5%



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(12.5% inhibition) and 57.6% (42.4% inhibition), respectively, relative to the untreated control. However, increasing the GSNO dose to 1 mM reduced DHT to 12.7% (87.3% inhibition) relative to untreated controls. Taken together, these results indicate that NO can inhibit the function of 5-α reductase and reduce the synthesis DHT from testosterone.

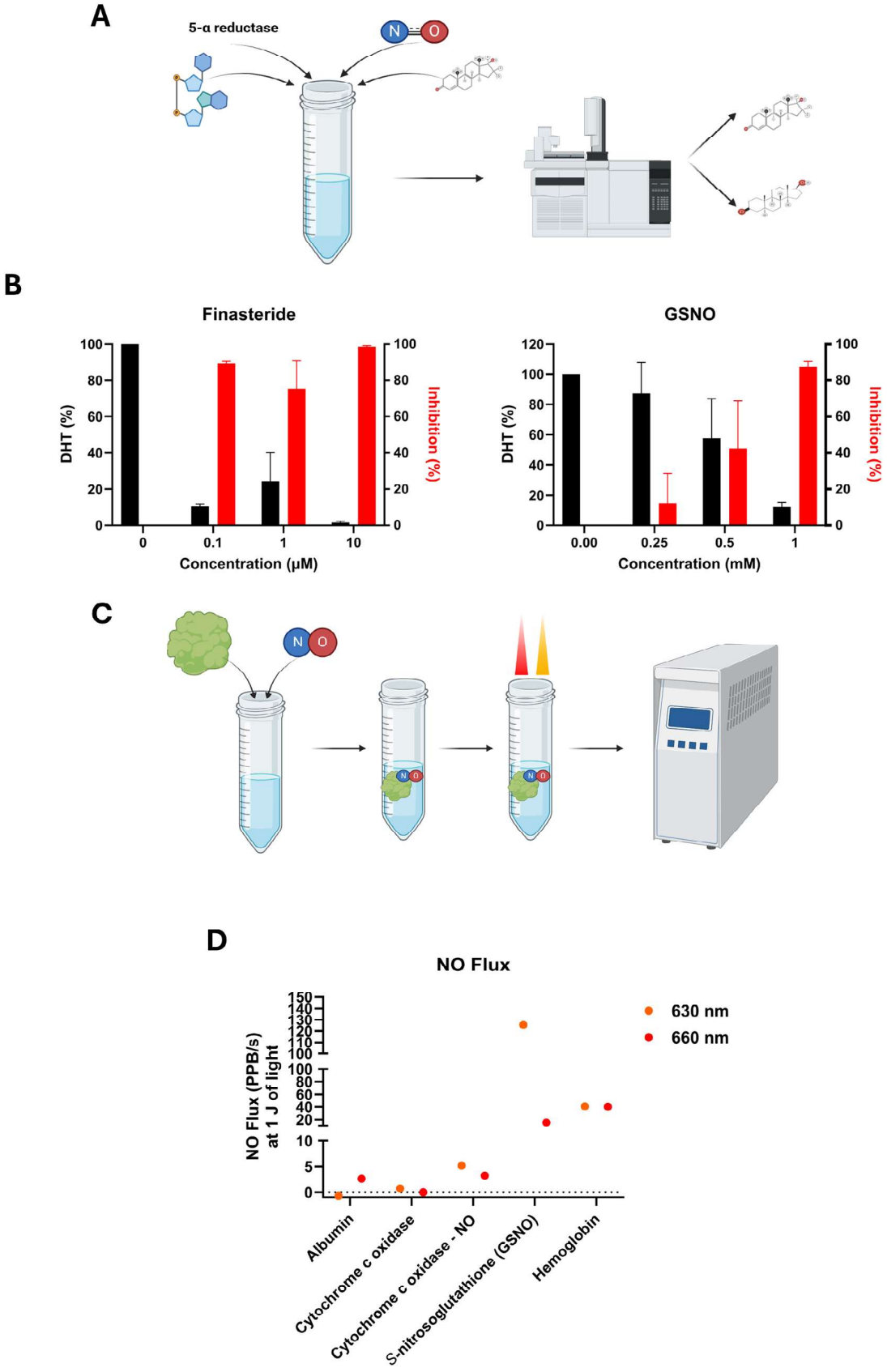


**FIGURE 2** | Legend on next page.



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### 3.2 | Red-Orange and Red Wavelengths of Light Promote Release of NO From Nitrosated/Nitrosylated Proteins

We next sought to determine how red-orange wavelengths of visible light induce release of NO from an array of proteins that are commonly nitrosated/nitrosylated. Nitrosated/nitrosylated albumin, cytochrome c oxidase, GSNO, and hemoglobin were illuminated with 630 nm or 660 nm LEDs for 20 min. NO release was measured via a chemiluminescent nitric oxide analyzer (Figure 2C). To account for the differences in power between 630 nm and 660 nm light, data are presented at 1 J of total light delivered (Figure 2D).

Each wavelength of light demonstrated the release of NO from nitrosated/nitrosylated proteins in vitro in a wavelength- and protein-dependent manner (Figure 2D). Specifically, 1 J of 630 nm wavelength light induced NO release from cytochrome c oxidase—NO, GSNO, and hemoglobin, but not albumin or cytochrome c oxidase. Conversely, 1 J of 660 nm light induced NO release from albumin, cytochrome c oxidase—NO, GSNO, and hemoglobin, but not cytochrome c oxidase. In general, both wavelengths induced similar levels of NO flux; however, 630 nm light induced more NO flux from GSNO than 660 nm light, while 660 nm light induced more NO flux from albumin. Taken together, these results indicate that both 630 and 660 nm light individually induce NO production via denitrosation/denitrosylation of proteins.

### 3.3 | LEDs, But Not Lasers, Induce Production of ROS in A549 Cells

After identifying that wavelengths of light in the red-orange and red region of the visible light spectrum induce NO release from free nitrosated/nitrosylated proteins, we next determined whether red and red–orange visible light could sufficiently induce biological responses within cells. To do this, we illuminated A549 epithelial cells with LEDs emitting 620, 660, or 620 + 660 nm light (dual) or lasers emitting 660 nm light and measured ROS production immediately following illumination with CellROX Deep Red reagent (Figure 3A).

In A549 cells, red-orange light stimulated ROS in an emitter- and wavelength-dependent manner (Figure 3B). Specifically, LEDs emitting 620 nm and 660 nm light individually induced ROS production in 80.3% ($p < 0.05$) and 89.0% ($p \leq 0.0005$) of cells (Figure 3C). LEDs emitting the dual wavelength combination induced ROS production in 92.5% ($p < 0.0001$) of cells immediately following illumination. Conversely, laser-emitted 660 nm light induced ROS production in 3.5% of cells, which was lower than homeostatic ROS production in unilluminated cells (5.8%). These results indicate that red and red-orange wavelengths of light emitted from LEDs induce cellular responses, while laser-emitted red light does not.

### 3.4 | Dual Wavelength LEDs Induce Production of NO in A549 Cells

The induction of ROS indicates that red and red–orange wavelengths of light elicit biological effects in epithelial cells. Based on the previous finding that red and red-orange wavelengths of light can release NO from nitrosated/nitrosylated proteins (Figure 2), we next sought to determine if these same wavelengths of light could induce the production of NO in cells. Thus, we illuminated A549 epithelial cells with LEDs emitting 620 nm, 660 nm, dual wavelengths or lasers emitting 660 nm light and measured NO production immediately following illumination with DAF-FM diacetate (Figure 3A).

In contrast with ROS production, NO production following illumination was limited (Figure 3B); few cells in the unilluminated, 620 nm LED, 660 nm LED, or 660 nm laser groups induced NO production. Specifically, 1.32% of unilluminated cells, 0.5% of 620 nm LED-illuminated cells, 2.7% of 660 nm LED-illuminated cells, and 0.9% of 660 nm laser-illuminated cells produced NO (Figure 3C). However, cells illuminated with LEDs emitting dual wavelengths induced consistent, detectable NO production with 44.1% ($p < 0.0001$) of cells producing NO. These results indicate that the limited NO production by red and red-orange wavelength illumination is augmented in the presence of two differing wavelengths of light.

### 3.5 | Dual Wavelength LEDs Induce Reactive Oxygen Species and Reduce DHT Conversion in 293T Cells

After determining that red and red-orange wavelengths of light from LEDs induce biological responses in epithelial cells, we next sought to determine if these wavelengths reduced concentrations of DHT following illumination in comparison to the FDA-approved finasteride. For these experiments, we utilized HEK 293T cells instead of A549 cells as A549 cells only express Type I 5-α reductase [17, 18], while HEK 293T cells express Type II 5-α reductase [19] and allow the use of finasteride as a positive control and comparator. In this experiment, we detected ROS production following illumination then assessed production of DHT following illumination or treatment with finasteride.

**FIGURE 2** | Red and red-orange wavelengths of light release NO from nitrosated/nitrosylated proteins, which inhibits DHT synthesis by 5-α reductase. (A) The 5-α reductase enzyme was isolated from human prostate tissue and incubated at 37°C with an excess of testosterone, NADPH, and GSNO or finasteride. The reaction was terminated with acetonitrile. DHT was extracted and its concentration determined via gas chromatography–mass spectrometry. (B) Data presented are the mean remaining DHT (%) and inhibition (%) relative to untreated samples. Data were collected across 2–3 independent experiments. (C) Proteins and the low molecular weight glutathione known to store NO in vivo were nitrosated/nitrosylated with commercially available NO-donating compounds. Following nitrosation/nitrosylation, the compounds were illuminated with red-orange (630 nm) or red (660 nm) wavelengths of light for 20 min. The amount of NO release was measured via chemiluminescence with a nitric oxide analyzer. (D) Data plotted are NO Flux at 1 J of light energy. Methods figure created with Biorender.com.



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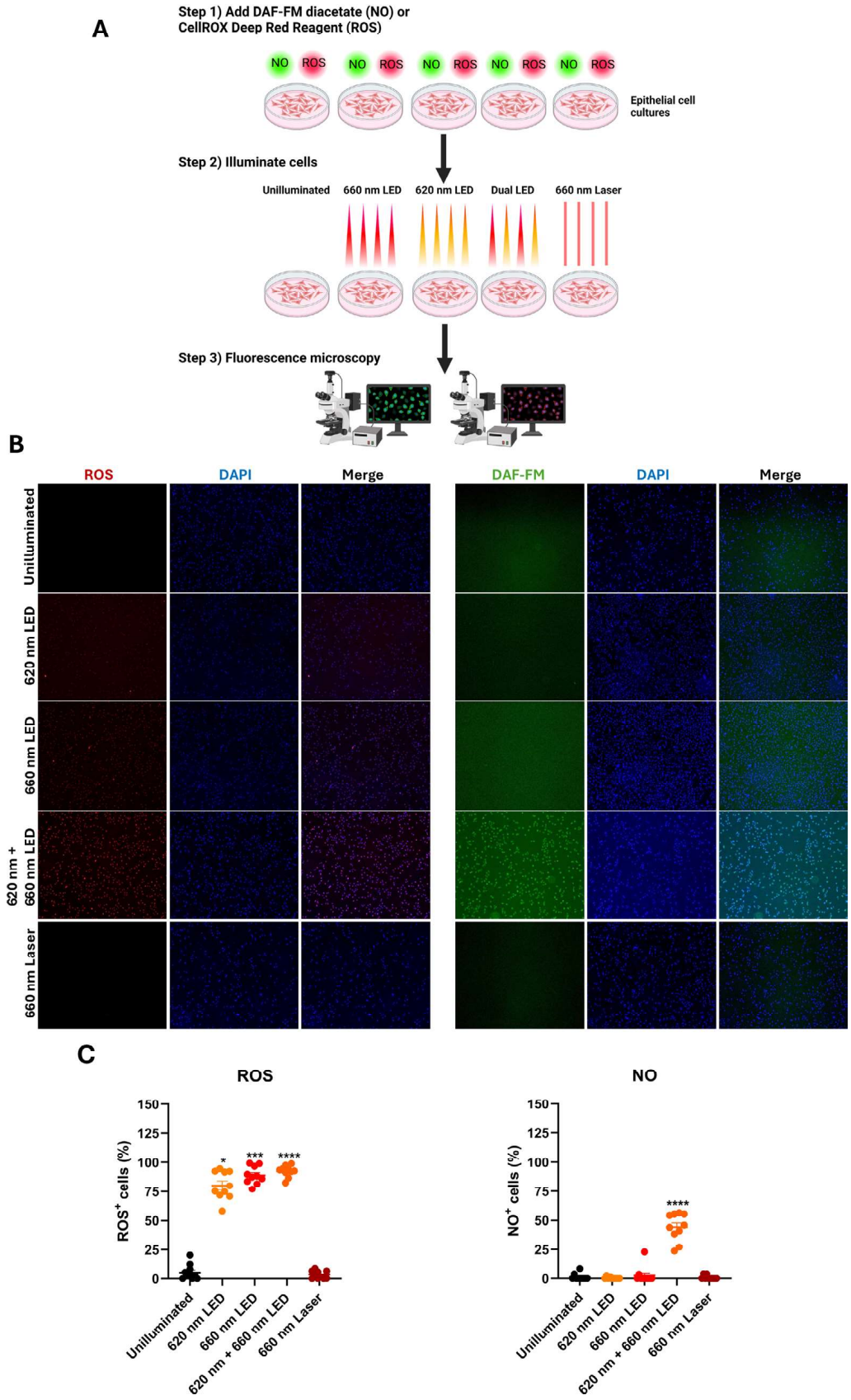


**FIGURE 3** | Dual wavelength LEDs stimulate production of NO and ROS in a synergistic manner. (A) DAF-FM diacetate and CellROX Deep Red reagent were added to determine production of NO and ROS, respectively. Cells were illuminated with LEDs and lasers for 10 and 6 min, respectively. ROS and NO production were measured immediately following illumination (0 h post-illumination). (B) Representative images of two independent experiments. (C) Data presented are mean percentage of cell expressing ROS or NO from two independent experiments. Methods figure created with Biorender.com.



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First, we measured ROS production in HEK 293T cells, illuminated with LEDs or lasers (Figure 4A). As we observed in A549 cells, only LED-emitted light stimulated production of ROS in HEK 293T cells. Conversely, there was no detectable ROS production in unilluminated or laser-illuminated HEK 293T cells. These results strongly corresponded to the observed ROS production in A549 cells.

Next, we illuminated HEK 293T cells with LEDs or lasers or treated with finasteride before adding testosterone to measure DHT synthesis at 2 hpi, 6 hpi, and 24 hpi (Figure 4B). DHT concentration was measured via competitive ELISA. At 24 hpi, dual LED wavelengths significantly reduced DHT concentration (0.20 ng/mL) relative to the unilluminated control (0.92 ng/mL, $p \leq 0.05$), corresponding to a 79.3% reduction in DHT (Figure 4C). Similarly, finasteride reduced DHT concentrations at each timepoint evaluated, though these differences were not statistically significant; the peak reduction by finasteride was approximately 40% at 2 hpi. The 620 nm LED demonstrated a trend for reduced DHT at 2 hpi and 6 hpi relative to unilluminated control, although these differences were not statistically significant. Conversely, the two 660 nm emitters (LED and laser) did not induce any reductions in DHT at any timepoint tested. These results demonstrate that illumination with dual wavelength LEDs induces a biological response that inhibits the conversion of testosterone to DHT in HEK 293T cells.

### 3.6 | LEDs Induce Reactive Oxygen Species in Primary Hair Follicle Dermal Papilla Cells

After showing that the dual LED wavelengths can inhibit the conversion of testosterone to DHT by 5-α reductase in an immortalized epithelial cell line, we finally sought to investigate the biological effect of LLLT in a translationally relevant model, primary human hair follicle dermal papilla cells (HFDPC). Thus, we examined ROS production and DHT synthesis by primary HFDPC (Figure 5).

First, we measured ROS production in HFDPC illuminated with LEDs or lasers (Figure 5A). As we observed in A549 and HEK 293T cells, only LED-emitted light stimulated production of ROS in HFDPC. Specifically, 620 nm LEDs, 660 nm LEDs, and the dual LED wavelengths each induced detectable ROS; the relative intensity of ROS production was highest in the dual wavelength LED illumination group. Conversely, there was no detectable ROS in unilluminated HFDPCs or in HFDPCs illuminated with lasers emitting 660 nm. These results in primary HFDPCs strongly correspond with the ROS production observed in both immortalized epithelial cell lines.

Next, we illuminated HFDPCs with LEDs, illuminated with lasers, or treated with finasteride before adding testosterone to measure DHT synthesis at 2 hpi, 6 hpi, and 24 hpi. DHT concentration was measured via competitive ELISA (Figure 5B). In contrast to HEK 293T cells, we did not observe any trends or statistically significant changes in any treatment groups, including the finasteride positive control, relative to the unilluminated control group (Figure 5B). These results indicate that in primary HFDPCs in vitro, red and red–orange wavelengths of light and finasteride do not reduce DHT synthesis despite the consistent induction of ROS by LED-emitted light. Because the finasteride positive control did not show a decrease in DHT, it is likely that LED emitted light lowers DHT through lowering Type II 5-α reductase activity, which is not expressed in culture HFDPCs, but is expressed in vivo in HFDPCs [14]. With the induction of ROS by LED-emitted light, it is probable that in a system where HFDPCs express both type I and type II 5-α reductase, LED-emitted light would lead to a reduction of DHT.

## 4 | Discussion

Our understanding of the mechanisms driving LLLT-based treatment of AGA to date remains largely theoretical. Numerous factors have been suggested, including NO production [9, 20], increased cell proliferation [5], reduced inflammation [5], and modulated gene expression [9, 21]. Despite the growing interest in noninvasive approaches to treat AGA, there are limited studies available that have investigated LLLT against a driver of AGA: the synthesis of DHT from testosterone to DHT by 5-α reductase. Previous research has indicated that LLLT can modulate 5-α reductase expression [13] and can theoretically inhibit DHT synthesis. The present study builds on this foundation; in this study, we show that LLLT with LED-emitted dual wavelengths of red and red–orange light inhibits the synthesis of DHT by 5-α reductase. Notably, the observed biological effects were strongest in dual LED wavelengths compared to individual wavelengths, suggesting a synergistic effect of 620 and 660 nm light. Further, this study suggests that LED-based LLLT primarily functions through inhibition of Type II 5-α reductase and has minimal impact on Type I 5-α reductase. Within the cell lines utilized in this study, A549 cells express Type I 5-α reductase [17, 18], HEK 293T cells express Type II 5-α reductase [19], and HFDPCs produce both Types I and II 5-α reductase; however, HFDPCs produce only Type I 5-α reductase in vitro [14, 15]. Finasteride, a known Type II 5-alpha reductase inhibitor [2, 22], inhibited the synthesis of DHT in HEK 293T cells (Figure 4C). However, finasteride had no inhibition activity in scalp-derived HFDPCs in the present study (Figure 5B) likely owing to the loss or low expression of Type II 5-α reductase expression in primary HFDPC cultures in vitro [14, 15]. Similar trends were observed with dual wavelength LED illumination, providing evidence that LED-LLLT inhibits Type II 5-α reductase. Despite the lack of DHT synthesis inhibition observed in the primary HFDPC model, it is notable that strong ROS activation was observed, suggesting that the LED-based LLLT approach will inhibit DHT synthesis in translational models which express Type II 5-α reductase, including immortalized dermal papilla cells [23, 24], ex vivo hair follicles [25], or three-dimensional organoid cultures [11, 26].

In this study, the strongest biological impacts were observed in cells treated with dual wavelength LEDs. Indeed, aside from ROS production, individual wavelengths of light did not induce consistent levels of NO or inhibit DHT synthesis regardless of the emitter type used. A key photoacceptor within human skin, cytochrome c oxidase (CCO), has four separate chromophores that can absorb light. Notably, these chromophores absorb different wavelengths of light centered around 620, 680, 760, and 820 nm [27]. Simultaneous illumination with two wavelengths of light and the broader spectral range emitted by LEDs likely activates multiple CCO chromophores.



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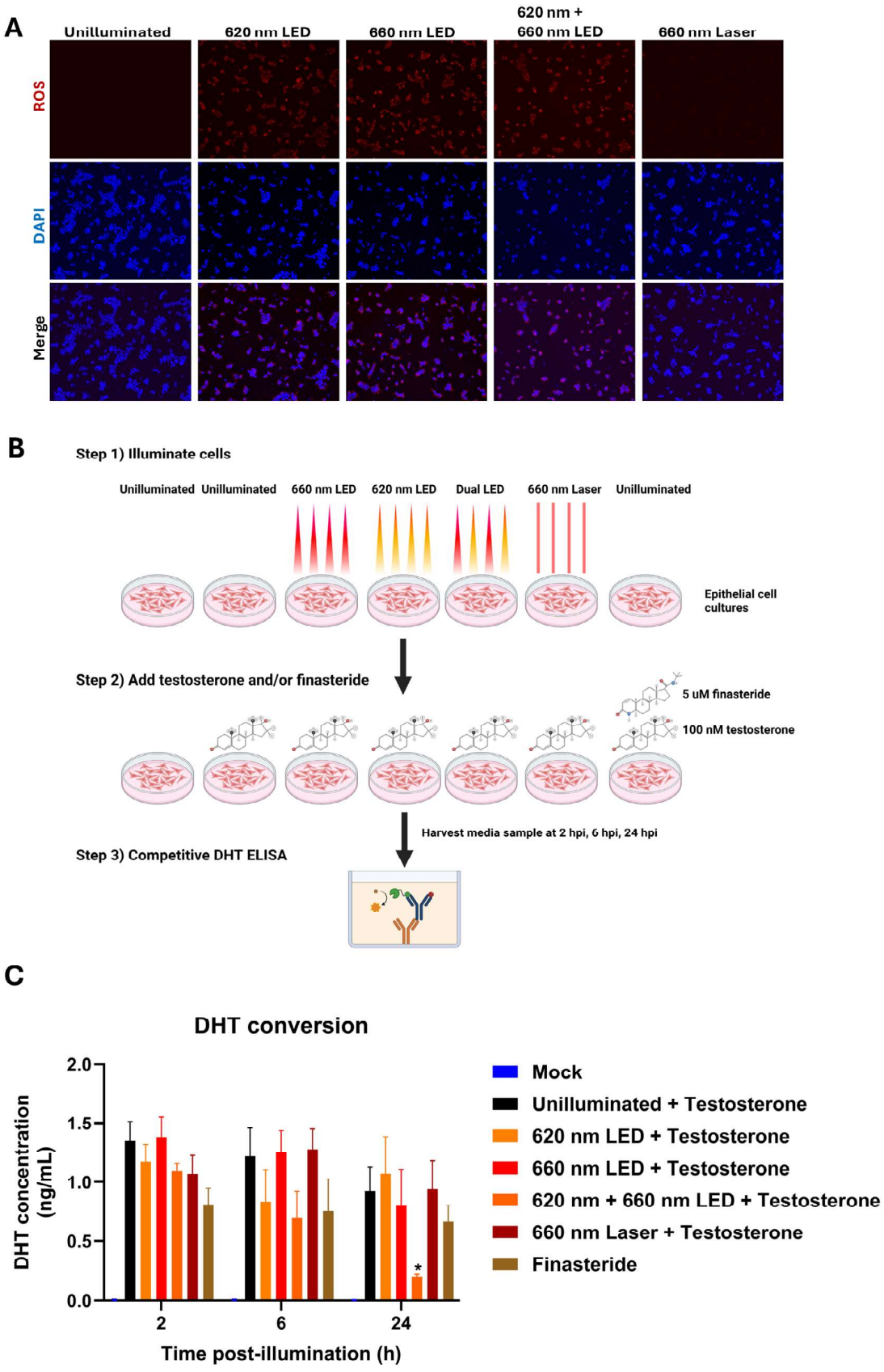


**FIGURE 4** | Dual wavelength LED lights inhibit conversion of testosterone to DHT in epithelial cells. (A) CellROX Deep Red Reagent was added to determine production of ROS. Cells were illuminated with LEDs and lasers for 10 and 6 min, respectively. ROS production was measured immediately following illumination (0 h post-illumination). Representative images of two independent experiments. (B) Cell cultures were illuminated with LED or Laser lights for 10 and 6 min, respectively. Immediately following illumination, testosterone was added to each plate. Samples were harvested at 2 h, 6 h, and 24 h following illumination. DHT concentration was determined via competitive ELISA (Abcam). (C) Data presented are DHT concentration +/− SEM from four independent experiments. Methods figure created with Biorender.com.



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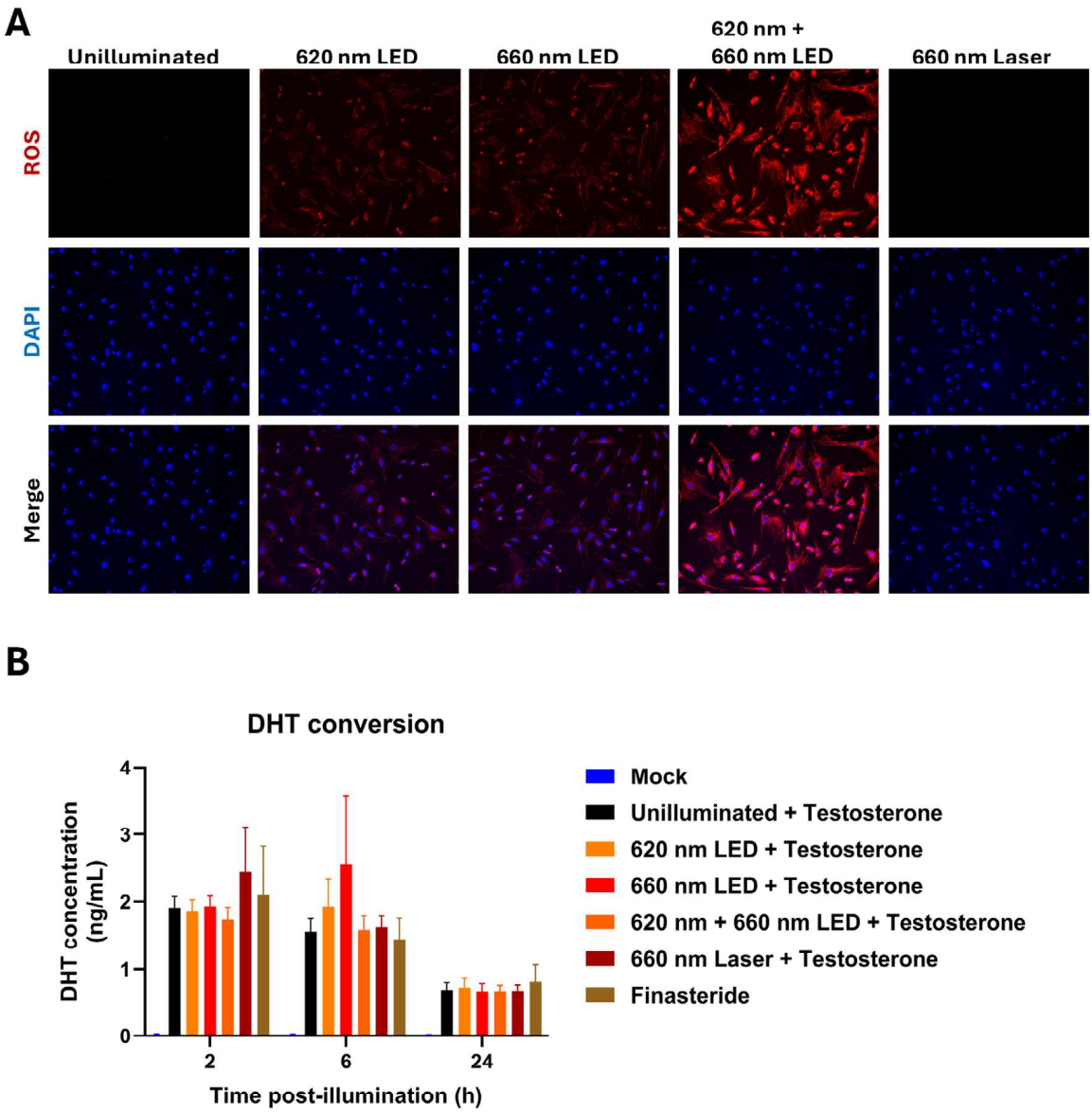


**FIGURE 5** | Dual wavelength LEDs stimulate ROS production but do not inhibit DHT synthesis in primary hair follicle dermal papilla cells. (A) CellROX Deep Red reagent was added to determine production of ROS. Cells were illuminated with LEDs and lasers for 10 and 6 min, respectively. ROS production was measured immediately following illumination (0 h post-illumination). Representative images of two independent experiments. (B) Cell cultures were illuminated with LED or Laser lights for 10 and 6 min, respectively. Immediately following illumination, testosterone was added to each plate. Samples were harvested at 2, 6, and 24 h following illumination. DHT concentration was determined via competitive ELISA (Abcam). Data presented are DHT concentration +/− SEM from four independent experiments.

In theory, this would displace more NO from CCO, resulting in greater NO-based biological effects downstream. Further, the observed preclinical findings in vitro with dual wavelengths of light have been supported in clinical study. In one prospective, double-blind, sham-controlled study, a dual wavelength LED (620 nm + 660 nm) device increased hair count by 26.3 hairs/cm$^2$ 16 weeks after enrollment [28]. In another study, 14 weeks of treatment with 655 and 780 nm light increased vertex hair density and anagen/telogen ratio [29]. Finally, a combination of 650 nm lasers with 630 and 660 nm LEDs reported increased mean hair thickness and hair density but did not report a change in overall appearance in the treated group [30]. Based on these preclinical findings and clinical reports, dual wavelength illumination increases the success of LLLT as a therapeutic for AGA.

This study also directly compares the biological responses in cells stimulated with LEDs and lasers. Within the context of LLLT, light from lasers and LEDs are considered to have substantial differences that are believed to improve biological responses by proponents of both modalities. For example, lasers produce collimated, coherent light, while LEDs produce noncoherent light ([6, 31]). Collimated, coherent light has been proposed to penetrate tissue deeper and stimulate mitochondria to induce biological response than noncoherent light [6]. However, these conclusions were reached prior to the development of modern LEDs; indeed, noncoherent light has been shown to induce comparable biological effects as coherent light in preclinical and clinical studies using modern LED technologies [6]. Our present study shows that in a wavelength- and power-matched system, LEDs induced stronger biological responses than lasers, including NO production, ROS



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production, and DHT synthesis inhibition. Thus, these results combined with results of past studies suggest that wavelength selection and sufficient power are more critical to induce physiologic effects than the type of emitter used.

AGA is the leading cause of hair loss in adult men and women [1]. The development of AGA is strongly associated with the synthesis of DHT from testosterone by 5-α reductase. Multiple pharmaceutical interventions have been shown to reduce DHT in vivo, corresponding to slowed hair loss and increased hair growth [1, 7, 22]. However, these systemically administered compounds have undesirable off-target effects that can skew the benefit–risk profile in individuals [22]. Given the demonstrated success in targeting 5-α reductase to reduce hair loss, localized treatments that offer similar benefits while reducing off-target risks are an intriguing option. The present study investigated one of these options, LLLT, which utilizes targeted wavelengths of light to induce a biological response. Our study demonstrates that red and red–orange wavelengths of light induces NO release from proteins in vitro, that NO inhibits 5-α reductase activity, and that a combination of red and red–orange light induces biological responses that reduce DHT synthesis in epithelial cells. Taken together, this study strengthens our understanding of the mechanisms behind LLLT treatment of AGA.

### Conflicts of Interest

Authors J. K., N. J., D. S., P. J. D., T. M. W., K. C., N. S. conducted this work on behalf of REVIAN Inc. through their employment relationship with KNOW Bio LLC (parent company of REVIAN) and may have ownership interests in one or both companies. All patents and applications arising from these findings are assigned to KNOW Bio LLC.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### References


1. C. H. Ho, T. Sood, and P. M. Zito, "Androgenetic Alopecia," in *StatPearls* (Treasure Island, FL: StatPearls Publishing, 2024), https://www.ncbi.nlm.nih.gov/books/NBK430924/.

2. A. Courtney, D. Triwongwarant, I. Chim, S. Eisman, and R. Sinclair, "Evaluating 5 Alpha Reductase Inhibitors for the Treatment of Male Androgenic Alopecia," *Expert Opinion on Pharmacotherapy* 24, no. 18 (2023): 1919–1922, https://doi.org/10.1080/14656566.2023.2280630.

3. G. Bartsch, R. S. Rittmaster, and H. Klocker, "Dihydrotestosterone and the Concept of 5α–Reductase Inhibition in Human Benign Prostatic Hyperplasia," *European Urology* 37, no. 4 (2000): 367–380, https://doi.org/10.1159/000020181.

4. R. V. Clark, D. J. Hermann, G. R. Cunningham, T. H. Wilson, B. B. Morrill, and S. Hobbs, "Marked Suppression of Dihydrotestosterone in Men With Benign Prostatic Hyperplasia by Dutasteride, a Dual 5α-Reductase Inhibitor," *Journal of Clinical Endocrinology & Metabolism* 89, no. 5 (2004): 2179–2184, https://doi.org/10.1210/JC.2003-030330.

5. H. Chung, T. Dai, S. K. Sharma, Y. Y. Huang, J. D. Carroll, and M. R. Hamblin, "The Nuts and Bolts of Low-Level Laser (Light) Therapy," *Annals of Biomedical Engineering* 40, no. 2 (2012): 516–533, https://doi.org/10.1007/S10439-011-0454-7.

6. V. Heiskanen and M. R. Hamblin, "Photobiomodulation: Lasers vs Light Emitting Diodes?," *Photochemical & Photobiological Sciences: Official Journal of the European Photochemistry Association and the European Society for Photobiology* 17, no. 8 (2018): 1003–1017, https://doi.org/10.1039/C8PP00176F.

7. A. K. Gupta, R. R. Mays, M. S. Dotzert, S. G. Versteeg, N. H. Shear, and V. Piguet, "Efficacy of Non-Surgical Treatments for Androgenetic Alopecia: A Systematic Review and Network Meta-Analysis," *Journal of the European Academy of Dermatology and Venereology* 32, no. 12 (2018): 2112–2125, https://doi.org/10.1111/JDV.15081.

8. S. Lueangarun, P. Visutjindaporn, Y. Parcharoen, P. Jamparuang, and T. Tempark, "A Systematic Review and Meta-Analysis of Randomized Controlled Trials of United States Food and Drug Administration-Approved, Home-Use, Low-Level Light/Laser Therapy Devices for Pattern Hair Loss: Device Design and Technology," *Journal of Clinical and Aesthetic Dermatology* 14, no. 11 (2021): E64.

9. P. Avci, G. K. Gupta, J. Clark, N. Wikonkal, and M. R. Hamblin, "Low-Level Laser (Light) Therapy (LLLT) for Treatment of Hair Loss," *Lasers in Surgery and Medicine* 46, no. 2 (2014): 144–151, https://doi.org/10.1002/LSM.22170.

10. M. R. Hamblin, "Photobiomodulation for the Management of Alopecia: Mechanisms of Action, Patient Selection and Perspectives," *Clinical, Cosmetic and Investigational Dermatology* 12 (2019): 669–678, https://doi.org/10.2147/CCID.S184979.

11. B. Roets, "Potential Application of PBM Use in Hair Follicle Organoid Culture for the Treatment of Androgenic Alopecia," *Materials Today Bio* 23 (2023): 100851, https://doi.org/10.1016/J.MTBIO.2023.100851.

12. K. Yang, Y. Tang, Y. Ma, et al., "Hair Growth Promoting Effects of 650 nm Red Light Stimulation on Human Hair Follicles and Study of Its Mechanisms via RNA Sequencing Transcriptome Analysis," *Annals of Dermatology* 33, no. 6 (2021): 553–561, https://doi.org/10.5021/AD.2021.33.6.553.

13. R. A. Weiss, D. McDaniel, R. G. Geronemus, and M. A. Weiss, "LED Photobiomodulation Induced Hair Growth Stimulation," in *Lasers in Surgery and Medicine* (Hoboken, NJ: John Wiley & Sons, Inc, 2005), 27.

14. Y. Asada, T. Sonoda, M. Ojiro, et al., "5α-Reductase Type 2 Is Constitutively Expressed in the Dermal Papilla and Connective Tissue Sheath of the Hair Follicle in Vivo but Not During Culture In Vitro," *Journal of Clinical Endocrinology & Metabolism* 86, no. 6 (2001): 2875–2880, https://doi.org/10.1210/JCEM.86.6.7545.

15. S. Liu and H. Yamauchi, "Different Patterns of 5α-Reductase Expression, Cellular Distribution, and Testosterone Metabolism in Human Follicular Dermal Papilla Cells," *Biochemical and Biophysical Research Communications* 368, no. 4 (2008): 858–864, https://doi.org/10.1016/J.BBRC.2008.01.130.

16. C. Amaral, S. C. Cunha, J. O. Fernandes, et al., "Development of a New Gas Chromatography–Mass Spectrometry (GC–MS) Methodology for the Evaluation of 5α-Reductase Activity," *Talanta* 107 (2013): 154–161, https://doi.org/10.1016/J.TALANTA.2012.12.045.

17. F. G. Kapp, A. Sommer, T. Kiefer, G. Dölken, and B. Haendler, "5-Alpha-Reductase Type I (SRD5A1) is up-Regulated in Non-Small Cell Lung Cancer but Does Not Impact Proliferation, Cell Cycle Distribution or Apoptosis," *Cancer Cell International* 12, no. 1 (2012): 1–11, https://doi.org/10.1186/1475-2867-12-1/FIGURES/9.

18. P. R. Provost, C. H. Blomquist, C. Godin, et al., "Androgen Formation and Metabolism in the Pulmonary Epithelial Cell Line A549: Expression of 17β-Hydroxysteroid Dehydrogenase Type 5 and 3α-Hydroxysteroid Dehydrogenase Type 3," *Endocrinology* 141, no. 8 (2000): 2786–2794, https://doi.org/10.1210/ENDO.141.8.7589.

19. B. U. Panter, J. Jose, and R. W. Hartmann, "5alpha-Reductase in Human Embryonic Kidney Cell Line HEK293: Evidence for Type II Enzyme Expression and Activity," *Molecular and Cellular Biochemistry* 270, no. 1–2 (2005): 201–208, https://doi.org/10.1007/S11010-005-4508-8.

20. S. Farivar, T. Malekshahabi, and R. Shiari, "Biological Effects of Low Level Laser Therapy," *Journal of Lasers in Medical Sciences* 5, no. 2 (2014): 58.




18640648, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jbio.202400388 by Jacob Kocher , Wiley Online Library on [13/12/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

21. J. K. Pillai and V. Mysore, "Role of Low-Level Light Therapy (LLLT) in Androgenetic Alopecia," *Journal of Cutaneous and Aesthetic Surgery* 14, no. 4 (2021): 385–391, https://doi.org/10.4103/JCAS.JCAS_218_20.

22. B. H. Salisbury, S. W. Leslie, and P. Tadi, "5α-Reductase Inhibitors," in *StatPearls* (Treasure Island, FL: StatPearls Publishing, 2024), https://www.ncbi.nlm.nih.gov/books/NBK555930/.

23. E. G. Y. Chew, J. H. J. Tan, A. W. Bahta, et al., "Differential Expression Between Human Dermal Papilla Cells From Balding and Non-Balding Scalps Reveals New Candidate Genes for Androgenetic Alopecia," *Journal of Investigative Dermatology* 136, no. 8 (2016): 1559–1567, https://doi.org/10.1016/j.jid.2016.03.032.

24. M. H. Kwack, O. B. Hamida, M. K. Kim, M. K. Kim, and Y. K. Sung, "Establishment and Characterization of Matched Immortalized Human Frontal and Occipital Scalp Dermal Papilla Cell Lines From Androgenetic Alopecia," *Scientific Reports* 13, no. 1 (2023): 1–11, https://doi.org/10.1038/s41598-023-48942-4.

25. C. T. Tan, C. Y. Lim, and K. Lay, "Modelling Human Hair Follicles—Lessons From Animal Models and Beyond," *Biology* 13, no. 5 (2024): 312–325, https://doi.org/10.3390/BIOLOGY13050312.

26. S. Ntshingila, N. P. Khumalo, M. Engel, and A. T. Arowolo, "An Appraisal of Laboratory Models of Androgenetic Alopecia: A Systematic Review," *Skin Health and Disease* 1, no. 2 (2021): e15, https://doi.org/10.1002/SKI2.15.

27. T. I. Karu, "Multiple Roles of Cytochrome c Oxidase in Mammalian Cells Under Action of Red and IR-A Radiation," *IUBMB Life* 62, no. 8 (2010): 607–610, https://doi.org/10.1002/IUB.359.

28. M. S. Nestor, G. Ablon, A. Gade, H. Han, and D. L. Fischer, "Treatment Options for Androgenetic Alopecia: Efficacy, Side Effects, Compliance, Financial Considerations, and Ethics," *Journal of Cosmetic Dermatology* 20, no. 12 (2021): 3759–3781, https://doi.org/10.1111/JOCD.14537.

29. S. S. Kim, M. W. Park, and C. J. Lee, "Phototherapy of Androgenetic Alopecia With Low Level Narrow Band 655-Nm Red Light and 780-Nm Infrared Light," *Journal of the American Academy of Dermatology* 56, no. 2 (2007): AB112.

30. H. Kim, J. W. Choi, J. Y. Kim, J. W. Shin, S. J. Lee, and C. H. Huh, "Low-Level Light Therapy for Androgenetic Alopecia: A 24-Week, Randomized, Double-Blind, Sham Device-Controlled Multicenter Trial," *Dermatologic Surgery* 39, no. 8 (2013): 1177–1183, https://doi.org/10.1111/DSU.12200.

31. W. S. Kim and R. Glen Calderhead, "Is Light-Emitting Diode Phototherapy (LED-LLLT) Really Effective?," *Laser Therapy* 20, no. 3 (2011): 205–215, https://doi.org/10.5978/ISLSM.20.205.



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