

Research Article

Glutathione Combined with Punica granatum and Acerola Cherry Extracts reduced Melanin accumulation

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Received: 12 August 2021; **Accepted:** 19 August 2021; **Published:** 14 September 2021

Citation: Yung-Kai Lin, Yung-Hao Lin, Yung-Hsiang Lin, Chi-Fu Chiang. Glutathione Combined with Punica granatum and Acerola Cherry Extracts reduced Melanin accumulation. Journal of Food Science and Nutrition Research 4 (2021): 240-248.

Abstract

Blue light was common in natural light and white light, and long-term blue light exposure caused skin damage. Glutathione (GSH) inhibited melanin production and brightened the skin. In addition, the high content of polyphenol in punica grantum and vitamin C in acerola cherry had whitening effects. The purpose of this study was to explore whether GSH formula that combined GSH, punica grantum, and acerola cherry can reduce melanin formation. Blue light to stimulate A375 cell line to produce melanin, used GSH formula to treat A375 cell line, analyzed melanin, tyrosinase activity, melanogenesis associated genes, and functional analysis in CCD-996SK by nCounter analysis. GSH formula significantly decreased the melanin content and

tyrosinase activity under blue light exposure. GSH formula also decreased melanogenesis associated genes. And nCounter analysis showed that GSH formula had antioxidant, anti-aging, DNA repair, and anti-inflammatory abilities. GSH formula supplemented with GSH, punica granatum, and acerola cherry extracts can reduce melanin formation, and accompanied by anti-oxidation, anti-aging, DNA repair, anti-inflammatory ability.

Keywords: Acerola cherry; Glutathione; Punica Granatum; Melanin

1. Introduction

The visible light with a shorter 600 nm wavelength can produce pigmentation and cause melanin formation. In particular blue light studies, some evidence already demonstrated the cutaneous photoaging effects in vitro and in vivo [1,2]. Especially non-pigmented epithelial cells, blue light can cause cell dysfunction by generating ROS on DNA, resulting in cellular aging, age-related pathologies, and cancer [3]. Therefore, preventing damage from exposure to blue light was an important strategy for a healthy lifestyle. Glutathione (GSH) was considered as the anti-aging strategy for preventing reactive oxygen species (ROS) accumulation. It was also acting as the oxidative stress marker in plasma, urine, and saliva measuring [4]. GSH was an antioxidant with a thiol compound, and the tripeptide structure of GSH contained cysteine, glutamic acid, and glycine, which can regulate the melanin generation pathway of the skin. Some evidence had confirmed the variety of melanogenesis mechanisms of glutathione. (e.g., pheomelanin synthesis stimulation, antioxidant effects, and melanogenic enzyme interference). Although the whitening capacity of GSH was still controversial [5], it had been widely used as additives in benefit supplements and cosmetics applications industry for anti-aging. In recent years, collagen supplements have been widely used in the nutritional intake of modern people. To increase the anti-oxidation efficacy in collagen-based supplements for blue light exposure against, the combination of GSH and natural plant extracts was a common anti-blue light strategy. The punica granatum was originating from the Middle East, and was rich in high content of polyphenols (ellagitannins, gallotannins, anthocyanins, and catechin) and commonly processed into juice, wine, and dietary ingredient [6]. These active components contributed to various biological protective effects, including anti-inflammatory [7], antibacterial [8], antiviral [9], anti-aging [10], and anticancer [11]. The punica granatum exhibited photo-irradiated damage inhibition activity in

human fibroblast cells [12]. In addition, acerola cherry was native to the southern American and Caribbean, also called West Indies cherry or Barbados cherry. This cherry-like berries is not a true cherry and has been used as a folk medicine for liver ailments, diarrhea, dysentery, and cold flu [13]. Until now, the extremely high content of acerola's vitamin C was serving as the hot ingredient in the healthy foods industry, which is around 50-100 times than orange or lemon. Besides, this fruit contains abundant phenolic compounds, including benzoic acid derivatives, carotenoids, anthocyanins, flavonoids, and phenylpropanoids [14]. Same as punica granatum, acerola cherry also performed a great value with an anti-oxidant effect. However, it was still unclear whether the combination of GSH combined with punica granatum and acerola cherry can improve melanin production. In this study, we used GSH formula that combined GSH, punica granatum, acerola cherry, and then blue light to stimulate A375 cell line to produce melanin. Using GSH formula to treat cell line, analyzed melanin, tyrosinase activity, melanogenesis associated genes, and functional analysis in CCD-996SK by nCounter analysis. Herein, we proved the potential of inhibit melanin formation of GSH formula combined with punica granatum and acerola extract in vitro.

2. Materials and Methods

2.1 Cell lines and Materials

Human melanocyte A375 (CRL-1619) and fibroblast CCD-996SK (CRL-1881) cell lines were derived from the American Type Culture Collection (Manassas, VA, USA), and culture in growth media (Gibco®; minimum essential media, 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin). RNA extraction kit (Genaid Biotech), nCounter platform (NanoString Technologies). Glutathione with Collagen Plus Food Supplement Capsule (650 mg; ingredients: L-glutathione, fish collagen, blueberries juice powder,

spinach powder, Punica grantum, Acerola berry extract, royal jelly, sorbital, silicon dioxide, magnesium stearate and calcium phosphate) was opened, the measured dose of ingredient powder was dissolved in liquid form and performed to experiment treatment. The GSH (Sigma-Aldrich) was purchased for experimental uses.

2.2 Melanin content measurement

The experiment was following our previous publication procedure [15]. The A375 melanocyte cells were performed in this experiment. We seeded this cell line for 1.5×10^5 cell numbers in 6-well culture plates per well under 37°C and 5% CO₂ condition. Then 24 hours later, we transfer the culture plate to UV chamber (8J/cm²) to mimic blue light exposure and change the medium with fresh 3 ml of DMEM medium containing 0.25 mg/ml kojic acid, or GSH formula 0.25 mg/ml fruit extract, or blank solvent as mock set. Cells were harvested after incubation at 37°C for 48 h. Rinse the collected cells with 1 × PBS twice and then resuspend in 200 µl of 1 × PBS. The total cell lysates were obtained by freeze-thaw in liquid nitrogen for 10 minutes and room temperature for 30 minutes. Then used 12,000 × g (Thermo Scientific™ Heraeus™ Fresco™ 17 Microcentrifuge, Langenselbold, Germany) centrifugation for collecting the cell lysates by 30 minutes. Suspend the pellets with 120 µl of 1 N NaOH in 60°C dry bath for 1 hour. Take 100 µl of samples to 96-well plate for spectrophotometric measurement at 405 nm. The amounts of melanin were calculated as: Relative melanin formation (%) = $(OD_{\text{sample}}/OD_{\text{control}}) \times 100\%$.

2.3 Tyrosinase activity assay

The experiment was following our previous publication procedure [15]. The A375 melanocyte cells were performed in this experiment. We seeded this cell line for 1.5×10^5 cell numbers in 6-well culture plates per

well under 37°C and 5% CO₂ condition..After that, transfer the culture plate to UV chamber (8J/cm²) and change the medium with fresh 3 ml of DMEM medium containing 0.25 mg/ml kojic acid, or GSH formula 0.25 mg/ml fruit extract, or blank solvent as mock set. Cells were harvested after incubation at 37°C for 48 h. Rinse the collected cells with 1 × PBS twice and then resuspend in 200 µl of 1 × PBS. The cell lysates were obtained by freeze-thaw in liquid nitrogen for 10 min and room temperature for 30 minutes. Collect the pellets of cell lysates by centrifugation at 12,000 × g (Thermo Scientific™ Heraeus™ Fresco™ 17 Microcentrifuge, Langenselbold, Germany) for 30 minutes. After protein concentration determination. Perform the same concentration protein in a 96-well microtiter plate and adding total volume to the 90 µL. Protect from light and add 10 µL L-Dopa for 20 mins action response. After liquid becomes dark status, measure OD 450 for melanin content by ELISA reader.

2.4 Analysis of mRNA expression

1.5×10^5 CCD-966SK cells in 2 ml of the media with 0.25mg/ml of GSH formula were seeded in each well of 6-well plates and incubated for 24 hours. Then, we collected the cells and used the RNA extraction kit for RNA collection. Finally, we adjusted the RNA concentration to 75 ng/µL for mRNA expression analysis. The mRNA expression level was analyzed by qPCR. Primer sequence: TYR (F: CTCAAAGCAGCATGCACAAT/R: GCCCAGATCTTTGGATGAAA), TYRP1(F: GACACGCCTCCTTTTTATTCCA/R: ATGGGTTTGTCCCCCTGTTC), MC1R (F: CATCATCGACCCCCTCATCTAC/R: CAGGAACCAGACCACACAATATCA), MITF (F:GCCTCCAAGCCTCCGATAAG/R: GCACTCTCTGTTGCATGAACT)

Gene screening was performed nCounter gene expression platform (NanoString Technologies), and all

the operation steps were referenced by the recommend protocol and our previous publication [15,16].

2.5 Statistical Analysis

All values were expressed as mean ±SD. Between sample populations differences statistical result was determined by an unpaired two-tailed Student’s t-test. Statistical significance was considered at *p value* < 0.05.

3. Results

3.1 GSH formula decreased melanin content under blue light exposure

To clarify whether the GSH formula can reduce melanin, we used blue light to treat human melanoma

cell line A375 to induce melanin. Figure 1 showed that melanin content was increased to 113.7% under blue light exposure. Kojic acid (0.25 mg/ml) was considered as the whitening agent in cosmetics and used as the positive control in the anti-melanogenesis experiment [17]. The kojic acid treatment decreased the melanin content to 90.3%. GSH (0.25 mg/ml) treatment decreased the melanin content to 106%. Interestingly, GSH formula (0.25 mg/ml) treatment decreased the melanin content to 94.4%, and the inhibitory effect was similar to kojic acid. This result suggested that GSH formula can decrease melanin content under blue light exposure.

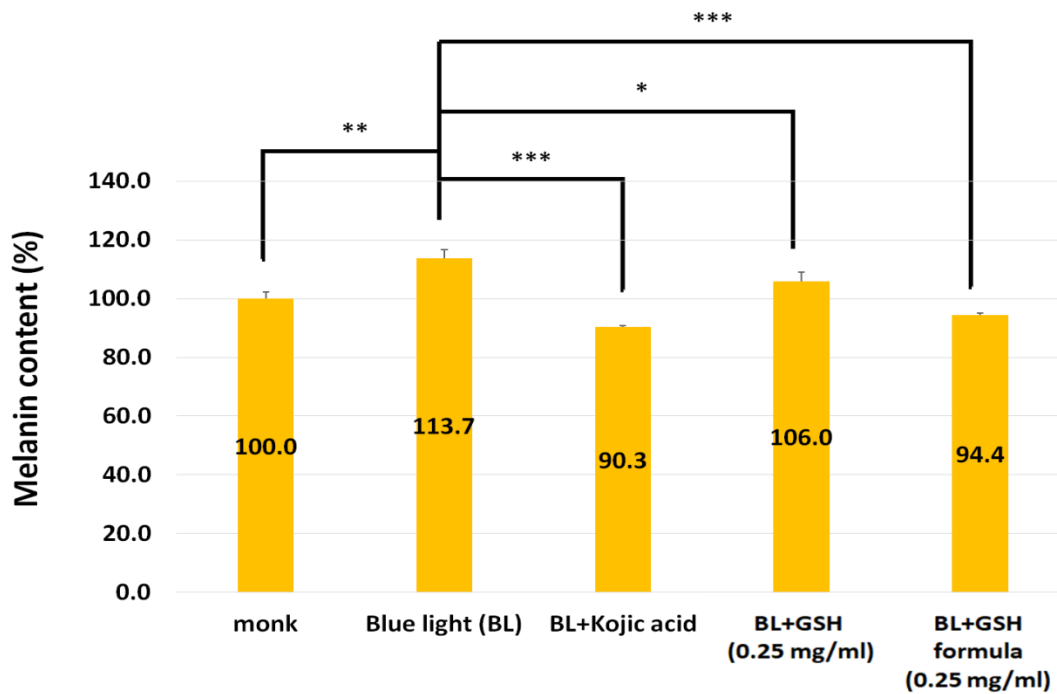


Figure 1: Effect of GSH formula reduces the melanin content compared to the GSH, kojic acid treatment only under blue light exposure. N=3, Data are means ± SD. Compared to the blue light exposed only (*, *p* < 0.05, **, *p* < 0.01; ***, *p* < 0.001)

3.2 GSH formula decreased tyrosinase activity and melanogenesis-associated gene expression.

Melanin was synthesized by tyrosinase [18]. To clarify whether the GSH formula can reduce tyrosinase activity, we treated the cells with GSH and then

measured the tyrosinase activity. The figure 2 showed that tyrosinase activity was increased to 138.3% under blue light exposure, and kojic acid treatment decreased tyrosinase activity to 84.6%. GSH formula treatment decreased the tyrosinase activity to 66.1%. This result suggested that GSH formula can decrease tyrosinase activity under blue light exposure. Next, we examined melanogenesis-associated gene expression, *TYRPI*

(Tyrosinase Related Protein 1), *MC1R* (Melanocortin 1 receptor), *MITF* (Melanocyte Inducing Transcription Factor), by Q-PCR. The figure 3 also showed that GSH formula can decrease melanogenesis-associated gene expression under blue light exposure. These results suggested that the GSH formula decreased tyrosinase activity and melanogenesis-associated gene expression.

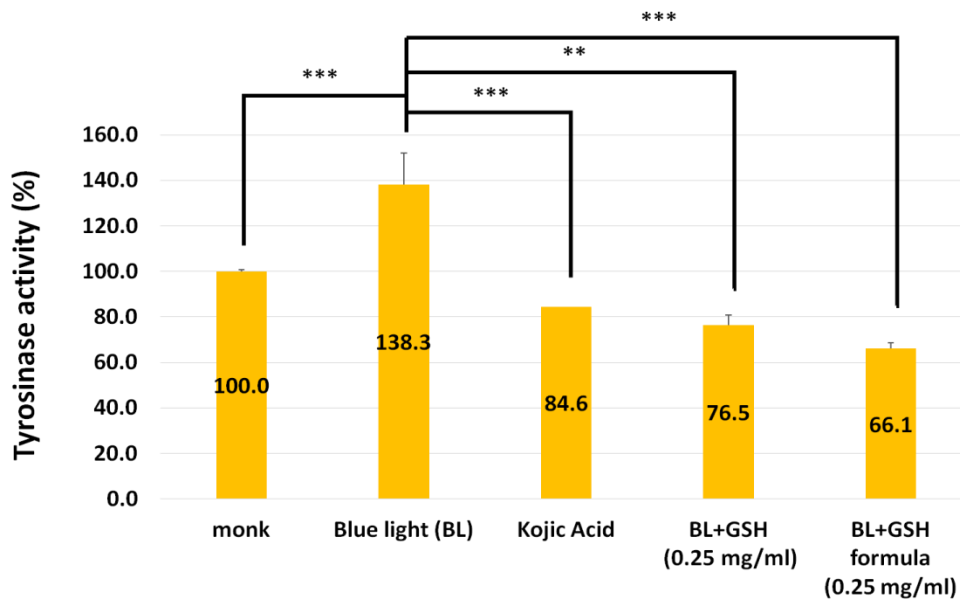


Figure 2: Effect of GSH formula reduces the tyrosinase activity compared to the kojic acid, GSH treatment only under blue light exposure. N=3, Data are means ± SD. Compared to the blue light exposed only (*, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$)

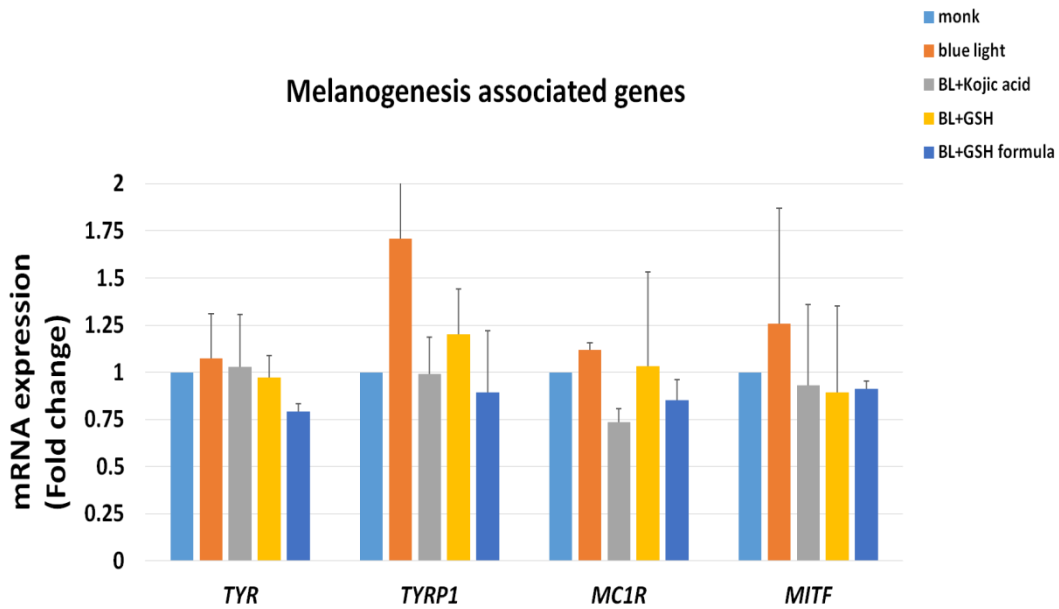


Figure 3: Melanogenesis genes mRNA expression effects of GSH formula treatment compared to the mock under blue light exposure. N=2, Data are means ± SD. Compared to the blue light exposed only. *TYR* (tyrosinase), *TYRP1* (Tyrosinase Related Protein 1), *MC1R* (Melanocortin 1 receptor), *MITF* (Melanocyte Inducing Transcription Factor)

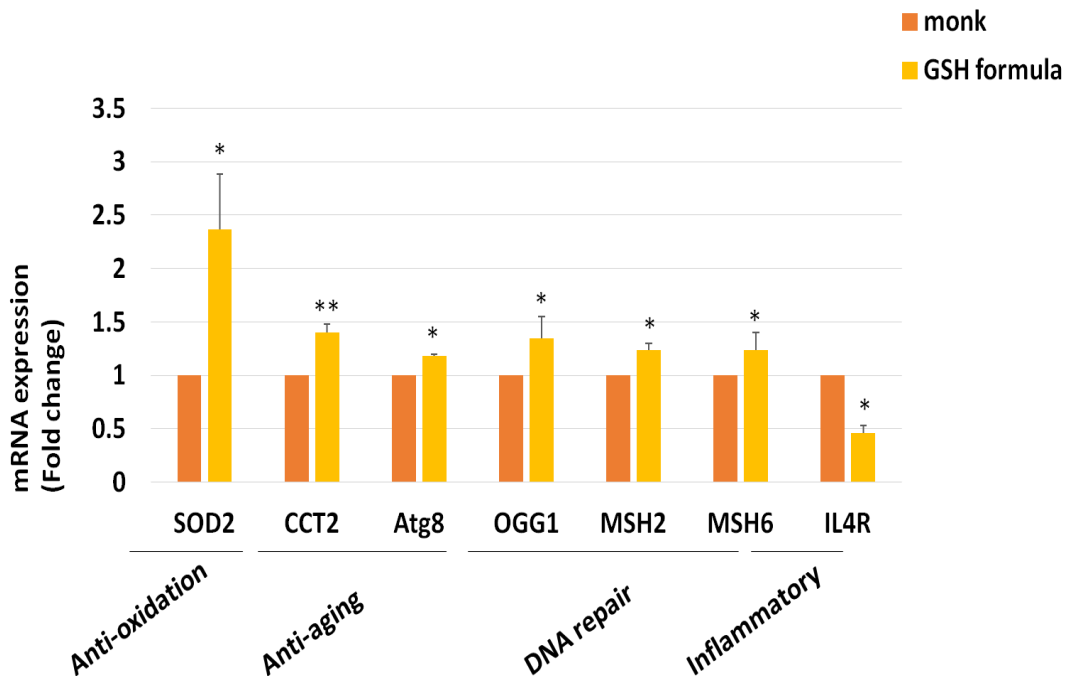


Figure 4: GSH formula treatment affected other functional genes mRNA by nCounter® screening. N=3, Data are means ± SD. Compared to the blue light exposed only (*, p < 0.05, **, p < 0.01; ***, p < 0.001) *SOD2* (Superoxide

dismutase 2), *CCT2* (Chaperonin Containing TCP1 Subunit 2), *Atg8* (Autophagy-related protein 8), *OGG1* (8-Oxoguanine glycosylase), *MSH2* (MutS Homolog 2), *MSH6* (mutS homolog 6), *IL4R* (interleukin-4 receptor).

3.3 The other functions of GSH formula

Next, we want to explore whether GSH formula had other functions. Using GSH formula to treat CCD-996SK cell lines, and then examined by nCounter® analysis. We examined *SOD2* (Superoxide dismutase 2) (anti-oxidation gene), *CCT2* (Chaperonin Containing TCP1 Subunit 2) and *Atg8* (Autophagy-related protein 8) (anti-aging gene), *OGG1* (8-Oxoguanine glycosylase), *MSH2* (MutS Homolog 2) and *MSH6* (mutS homolog 6) (DNA repair gene), and *IL4R* (interleukin-4 receptor) (inflammatory gene). The figure 4 showed that GSH formula can increase *SOD2*, *CCT2*, *Atg8*, *OGG1*, *MSH2* and *MSH6* gene expression, and decrease *IL4R*. Thus, GSH formula had antioxidant, anti-aging, DNA repair, and anti-inflammatory abilities.

4. Discussion

At the beginning of artificial light was available, the pressure of blue light exposure was around us. More and more evidence revealed the phototoxic for the skin damage and retina. Daily supplement intake is used to common habits of modern human style. Nowadays, GSH combined with vitamin C was regarded as a synergistic improvement of anti-oxidative efficacy [19]. In this study, we demonstrated GSH formula skin-lightening effects combined with high polyphenols of punica granatum ferments and high vitamin C of acerola cherry extract. Previous evidence suggested phenolics can increase the intrinsic GSH levels of subjects [20]. In addition, naturally occurring in vegetables, fruits, and plant-derived polyphenols also performed the coordinated regulation with GSH and its related enzymes [21]. More precisely, phenolic acids combined with GSH have synergistic effects in anti-oxidation [22]. This evidence indicated our GSH formula combined with high polyphenols of punica granatum

ferments is a great strategy for enhancing the anti-oxidant effects of glutathione. At the cellular level, senescence is a central hallmark of the aging process, and the senescence marker protein-30 (SMP30) expression was decreased during aging in zebrafish [23]. And acerola juice suppresses UVB-induced skin pigmentation efficacy has been provided in gluconolactonase SMP30 knockout hairless mice model [24]. In japan study also suggested acerola juice intake decrease the vitamin C excretion through urine in healthy subjects, which is in correspondence to the bioavailability of vitamin C improvement [25]. This evidence was suggested our strategy for supplementing acerola cherry in GSH formula. Taking together, the composition of GSH formula was supplemented with punica granatum ferments, acerola cherry extract with glutathione. And further examined the anti-pigmentation efficacy of this formula also performed efficacy enhancement effects (Figure.1,2,3). Despite kojic acid performed and great skin lightening efficacy in the cosmetic industry, it has been considered as a carcinogen and revealed the chronic toxicity response from dietary administration in rats [26,27]. Our combination GSH formula can successfully enhance the anti-pigmentation effect than GSH mono-treatment and performed a great efficacy closed to kojic acid mono-treatment. Moreover, our GSH formula was combined with safety botanical supplements to lessen adverse effects. GSH has been reported which can facilitate collagen-mediated fibroblast contraction and prevent cell death effect in cell line findings [28]. In our nCounter® screening findings, GSH formula can regulate anti-oxidant, anti-aging, DNA-repair, and anti-inflammation associated gene significantly (Figure 4). This result suggested the GSH formula didn't only

prevent the blue light exposure induced melanogenesis, it also performed other anti-aging efficacy.

5. Conclusion

In this study, the GSH formula was examined for the anti-pigmentation efficacy by cell line analysis. To combined high polyphenols punica granatum ferments, and acerola cherry extract. This formula successfully enhanced the anti-pigmentation efficacy of the glutathione. In addition, the skin-lightening effects of collagen also facilitated by these supplements. And GSH formula also contained other anti-aging properties through regulating different mechanisms associated with genes by nCounter screening. We demonstrated the comprehensively against blue light exposure protective function and anti-aging potential of GSH formula.

Conflict of interests

The authors have declared that there is no conflict of interest regarding this research.

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