EGCG induces degradation of active folate in serum via H_2O_2 generation, while L-ascorbic acid effectively reverses this effect

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ARTICLE INFO

ABSTRACT

Empirical studies have indicated that excessive tea consumption may potentially decrease folate levels within the human body. The main active component in green tea, epigallocatechin gallate (EGCG), significantly reduces the concentration of 5-methyltetrahydrofolate (5-MTHF) in both solution and serum. However, our findings also demonstrate that the pro-degradation effect of EGCG on 5-MTHF can be reversed by L-ascorbic acid (AA). Subsequent investigations suggest that EGCG could potentially expedite the degradation of 5-MTHF by generating hydrogen peroxide. In summary, excessive tea intake may lead to reduced folate levels in the bloodstream, yet timely supplementation of AA could potentially safeguard folate from degradation.

1. Introduction

Green tea is a globally popular beverage consumed by a large population in countries like China, Japan, as well as parts of North Africa and the Middle East [1]. Epigallocatechin gallate (EGCG) is the most abundant catechin in green tea. Upon oral ingestion, the C_{max} of EGCG can reach 4.4 μM [2], with habitual tea consumers often exhibiting elevated levels [3]. EGCG has been proven to have various effects such as antioxidant [4], anti-tumor [5], anti-inflammatory [6], anti-atherosclerotic [7], lipid-lowering [8], and therapeutic properties against neurodegenerative diseases [9]. However, excessive tea consumption may also lead to some negative effects, such as reducing the circulating folate levels within the body. A study on the interaction between tea and folate in healthy individuals revealed a reduced bioavailability of folate when consumed simultaneously with green tea [10]. In clinical trials involving pregnant women, a high intake of green tea during pregnancy was found to be inversely correlated with serum folate levels [11]. Additionally, in comparison to non-tea-consuming women during periconception, tea-consuming women exhibited a

threefold increased risk of neural tube defects (NTD) in embryos [12].

Folates are a group of structurally similar B-vitamin compounds essential for human physiological and developmental functions. The human body needs to obtain folate from diet to meet its requirements. 5-Methyltetrahydrofolate (5-MTHF) is the active form of folate and the form that the body can use directly [13]. Other forms of folate, such as folic acid, 5-formyltetrahydrofolate, necessitate conversion into 5-MTHF within the body for effective utilization. Typically, the plasma levels of 5-MTHF in healthy individuals range from 3 to 30 ng/ml (6.5–65 nM) [14]. Deficiency in folates may increase the risks of NTD [15], megaloblastic anemia [16], and neurodegenerative diseases [17, 18]. Under physiological conditions, 5-MTHF exhibits instability and susceptibility to oxidation by reactive oxygen species (ROS), culminating to increased degradation. The introduction of H_2O_2 to PBS can facilitate the oxidative degradation of 5-MTHF [19,20]. Moreover, the introduction of hydroxyl radicals in phosphate buffer can expedite the degradation of 5-MTHF [21].

EGCG acts as a competitive inhibitor of the proton-coupled folate transporter (PCFT), hindering the uptake of folate by PCFT [22].

However, apart from PCFT, folate can also be taken up through folate receptors (FR) and reduced folate carriers (RFC) [23]. Despite EGCG's influence on PCFT, existing evidence does not confirm its direct impact on folate concentration within the body. In vitro experiments, such as in cell culture or in solution, EGCG exhibits instability, which is related to the concentration of EGCG. Structural alterations or degradation occurs. At low concentrations (μM), it primarily undergoes autoxidation [24], while at high concentrations (mM), it retains relative stability but undergoes isomerization [24,25]. However, EGCG is relatively stable in vivo, and endogenous antioxidant enzymes help it stabilize. Currently, there is no literature describing whether EGCG affects folate levels during these processes.

In this study, we found that EGCG induces the degradation of 5-MTHF in both solution and serum, and ʟ-ascorbic acid (AA) effectively maintains the concentration of 5-MTHF. Furthermore, we demonstrated that EGCG destabilizes 5-MTHF by generating H_2O_2, while AA preserved 5-MTHF stability by scavenging H_2O_2.

2. Materials and methods

2.1. Materials

EGCG (≥98 %) and 10 × phosphate buffer were purchased from Solarbio (Beijing, China), while L-ascorbic acid (>99 %) was purchased from Macklin (Shanghai, China). 5-MTHF (≥98 %) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China), and 5-MTHF-^{13}C5 (99 atom % ^{13}C, 95 % (CP)) was purchased from Sigma-Aldrich (Shanghai) Trading Co. Ltd (Shanghai, China). Hydrogen peroxide detection kit was purchased from Beyotime (Shanghai, China). EGCG was dissolved in methanol and diluted with PBS to prepare a 50 mM stock solution. L-ascorbic acid was dissolved in PBS to create an 11 % stock solution. The handling protocols for 5-MTHF and 5-MTHF-^{13}C5 solutions adhered to the methodologies detailed in Ref. [26].

2.2. LC/MS analysis

Following Cao Yunfeng's method [26], the preprocessing of solution and serum samples was conducted, and LC/MS was utilized for precise quantification of 5-MTHF. In brief, solid-phase extraction (SPE) was performed on the solution and serum samples, followed by analysis using the LC-MS 8060 NXCE mass spectrometry system. Samples were separated using a CORTECS T3 Column (2.7 μm, 2.1 mm*100 mm, 120 Å; Waters, Milford, MA, USA) with solvent A consisting of 0.1 % formic acid (v/v) and solvent B as methanol, following the same gradient and flow rate as detailed in Ref. [26], maintaining a column temperature of 40 °C. Detection was carried out using Multiple Reaction Monitoring (MRM) mode.

2.3. Sample preparations

When exploring the effect of EGCG on 5-MTHF, the EGCG stock solution was diluted with PBS and then mixed with the solution of 5-MTHF or serum to reach the final concentrations. In the case of examining the action of AA, it was first mixed with EGCG and then combined with the solution or serum of 5-MTHF. To validate the impact of H_2O_2, a 1 mM H_2O_2 standard solution was diluted with PBS and mixed with the solution of 5-MTHF or serum. Post preparation, the samples were kept at room temperature in a dark place and subjected to pretreatment at 0h, 6h, 24h, and 48h, followed by analysis through LC/MS.

The use of leftover mixed serum in the present study was approved by the Research Ethics Committee of Dalian Central Hospital, China. Serum samples were collected and mixed from 200 healthy adults. Their biochemical markers, including but not limited to Total Protein (TP), Albumin (ALB), Globulin (Glob), Hemoglobin (HB), and Red Blood Cell Count (RBC), were within normal ranges. All health data we obtained were acquired with their informed consent.

2.4. H₂O₂ quantification

Varying concentrations of EGCG (2 μM, 20 μM, 0.1 mM, and 1 mM) were prepared by diluting the 50 mM EGCG stock solution with PBS. Subsequently, these solutions were added to both solution and serum. We employed the guidelines outlined in the hydrogen peroxide detection kit to assess H_2O_2 concentration at designated time points of 0h, 6h, 24h, and 48h. In brief, the solution or serum was diluted with PBS, added to a 96-well plate, and then added with a H_2O_2 detection reagent. After shaking well, the mixture was incubated at room temperature for 30 min, and detecting the absorbance at 560 nm.

2.5. Statistical analysis

To minimize experimental errors, we included three parallel samples for each group in every experiment. To ensure the reliability of the results, each experiment was repeated at least 5 times. The data analysis and chart drawing were performed by GraphPad Prism 9. One-way ANOVA was employed to analyze multiple groups of data, and $p < 0.05$ was considered to be significant.

PD represents the stability of 5-MTHF. The PD at different time intervals from baseline was calculated as [27].

*PD = (Result at time - Baseline result) / Baseline result * 100,*

The baseline is the result at 0h for each experimental group.

3. Result

3.1. EGCG promotes the degradation of 5-MTHF

In the solution, EGCG induced the degradation of 5-MTHF, with varying degrees of impact at different concentrations (Fig. 1A). From 6 h onwards, 2 μM EGCG demonstrated pro-degradation effect, while 20 μM and 0.1 mM exhibit slightly stronger pro-degradation, and the effect of 1 mM being comparable to that of 2 μM. By 48 h, 2 μM, 20 μM, and 0.1 mM concentrations of EGCG still showed significant pro-oxidant, but the pro-degradation effect of 1 mM EGCG disappeared. PD can assist us in a more intuitive observation of the effects of EGCG. Before 24 h in the solution, there is a significant change in PD, while the change between 24 and 48 h is relatively small, indicating that the impact of EGCG on 5-MTHF gradually approaches a certain equilibrium (Fig. 1C). In lower concentrations of 5-MTHF PBS solution (3 nM), we also observed the pro-degradation effect of EGCG (Supplementary Fig. 1).

We employed the same method to investigate the impact of EGCG on 5-MTHF in serum. We found that within 48 h, 2 μM EGCG had no significant effect on 5-MTHF; However, 0.1 mM and 1 mM EGCG exhibited pro-degradation effects on 5-MTHF from 6 h onwards, and 20 μM EGCG caused a decrease in 5-MTHF concentration by 48 h. In comparison to the effects observed in the solution, EGCG displayed weaker pro-degradation effect in serum (Fig. 1C and D). With the increase in EGCG concentration, the change in PD becomes more noticeable in serum. However, under the effect of 1 mM EGCG, the change in PD between 24 and 48 h is relatively small.

3.2. L-ascorbic acid can counteract the anti-folate effect of EGCG

We have observed that in the solution, both 2 μM and 0.1 mM EGCG initiated the degradation of 5-MTHF within 6 h (Fig. 1E). When combined with AA, 2 μM EGCG maintained the stability of 5-MTHF (Fig. 2A–C). Combining 0.1 mM EGCG with different concentrations of AA resulted in distinct effects. At 0.001 % AA (56 μM), when combined with 0.1 mM EGCG, EGCG concentrations surpassed AA, showing a pro-degradation effect on 5-MTHF within 24 h (Fig. 2E); However, when combined with 0.1 % (5.6 mM) AA, where EGCG concentrations (2 μM) were notably lower than AA (56 μM), it protected 5-MTHF from
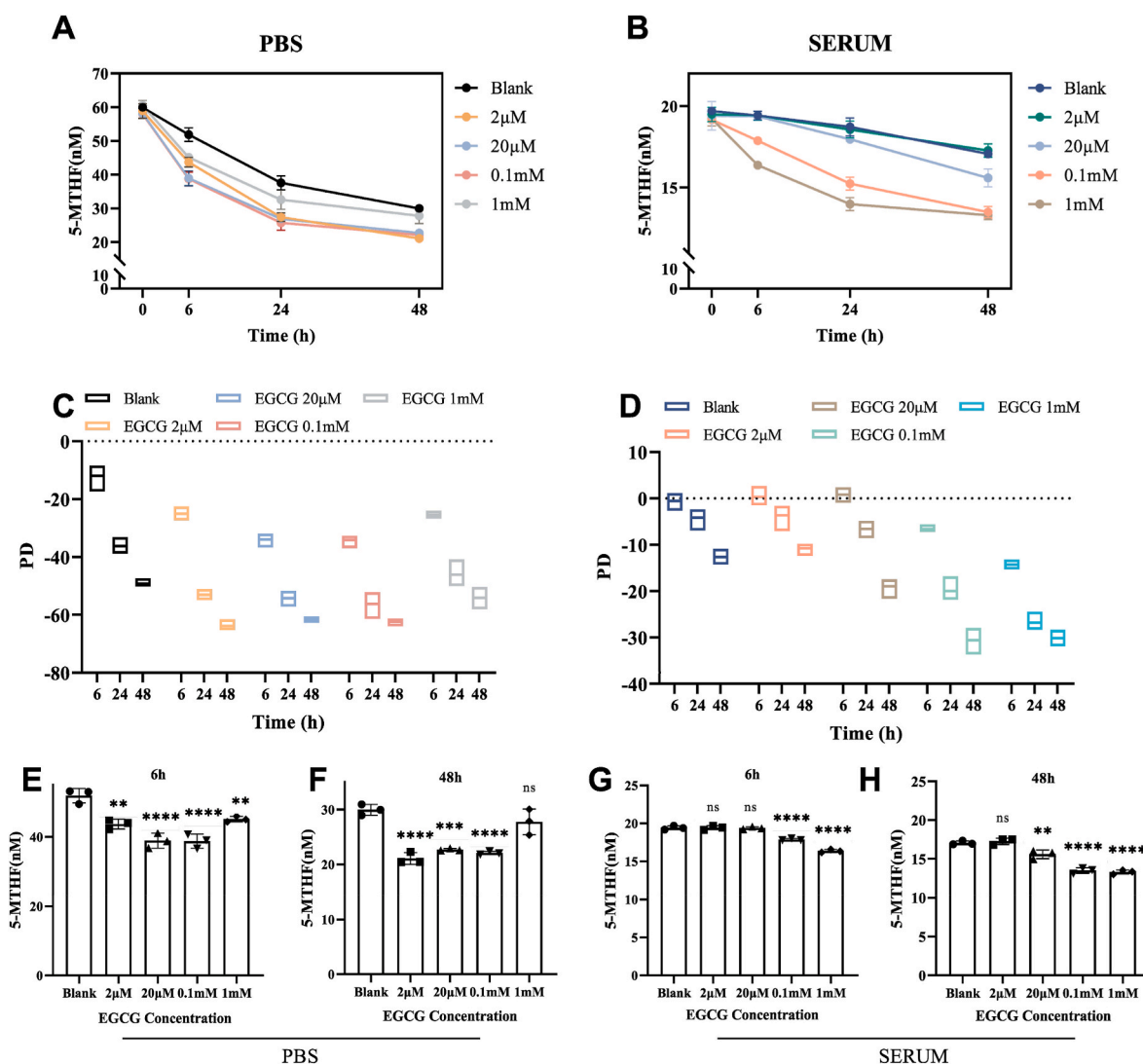
Fig. 1. EGCG's Impact on 5-MTHF in PBS and Serum. (A) Effect of EGCG on 5-MTHF (60 nM) in PBS. (B) Effect of EGCG on 5-MTHF in serum. (C) The PD of 5-MTHF treated with different concentration of EGCG at 6, 24 and 48 h in PBS, Data is represented as median (minimum to maximum). (D) The PD of 5-MTHF treated with different concentration of EGCG at 6, 24 and 48 h in serum, Data is represented as median (minimum to maximum). (E) EGCG effect on 5-MTHF in PBS at 6h. (F) EGCG effect on 5-MTHF in PBS at 48h. (E) EGCG effect on 5-MTHF in serum at 6h. (F) EGCG effect on 5-MTHF in serum at 48h. Values are mean ± SD, n = 3 per group, $^{ns}p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

degradation within 48 h (Fig. 2F).

In investigating the impact of EGCG on serum 5-MTHF, we discovered that 20 μM of EGCG exhibited pro-oxidant effect (Fig. 1H). When 20 μM EGCG was combined with AA, it counteracted the pro-degradation effect of EGCG (Fig. 2H). The combination of 0.1 mM EGCG with AA exhibited similar phenomena as in solution. When EGCG concentration (0.1 mM) exceeded AA (56 μM), AA couldn't effectively counteract EGCG's Pro-degradation effect on 5-MTHF. Conversely, when EGCG concentration (0.1 mM) was lower than AA (5.6 mM), AA counteracted EGCG's pro-degradation effect on 5-MTHF, exhibiting no significant difference compared to the blank group at 24 h (Fig. 2G).

3.3. EGCG induces the degradation of 5-MTHF by generating H_2O_2

Some studies indicate that EGCG undergoes autoxidation in solution, generating a semiquinone intermediate and subsequently producing H_2O_2 [28]. We detected the H_2O_2 generated by EGCG in both the solution and serum (Fig. 3A and B). In the solution, H_2O_2 rapidly generated and then gradually decreased, the concentration of H_2O_2 generated by 1 mM EGCG is relatively low. In serum, H_2O_2 generated by 2 μM

EGCG was near the baseline, H_2O_2 levels increased with the concentration of EGCG.

When EGCG was used in combination with AA, the generation of H_2O_2 was delayed or inhibited (Fig. 3C and D). In the solution, when 0.1 mM EGCG was combined with 0.001 % AA, where the concentration of EGCG exceeded that of AA, the production of H_2O_2 was delayed. When the concentration of AA (0.1 %) exceeded that of EGCG (0.1 mM), the generation of H_2O_2 was completely inhibited. The same phenomenon was observed in the serum.

To validate the impact of H_2O_2 on 5-MTHF, we introduced H_2O_2 into two matrices and used LC/MS to detect 5-MTHF concentration. The introduction of 30 μM H_2O_2 in the solution did not significantly affect the 5-MTHF concentration, whereas an increase in H_2O_2 concentration to 2 mM resulted in 5-MTHF degradation within 6 h (Supplementary Fig. 2A). In serum, 30 μM H_2O_2 showed negligible influence on 5-MTHF. Conversely, the addition of 2 mM H_2O_2 to serum immediately inhibited 5-MTHF levels (Supplementary Fig. 2B). We observed that after adding 2 mM H_2O_2 to the solution and serum, the degradation curve of 5-MTHF after 6 h remained parallel to the control group without H_2O_2 (Supplementary Figs. 2C and D). In other words, after partially degrading 5-
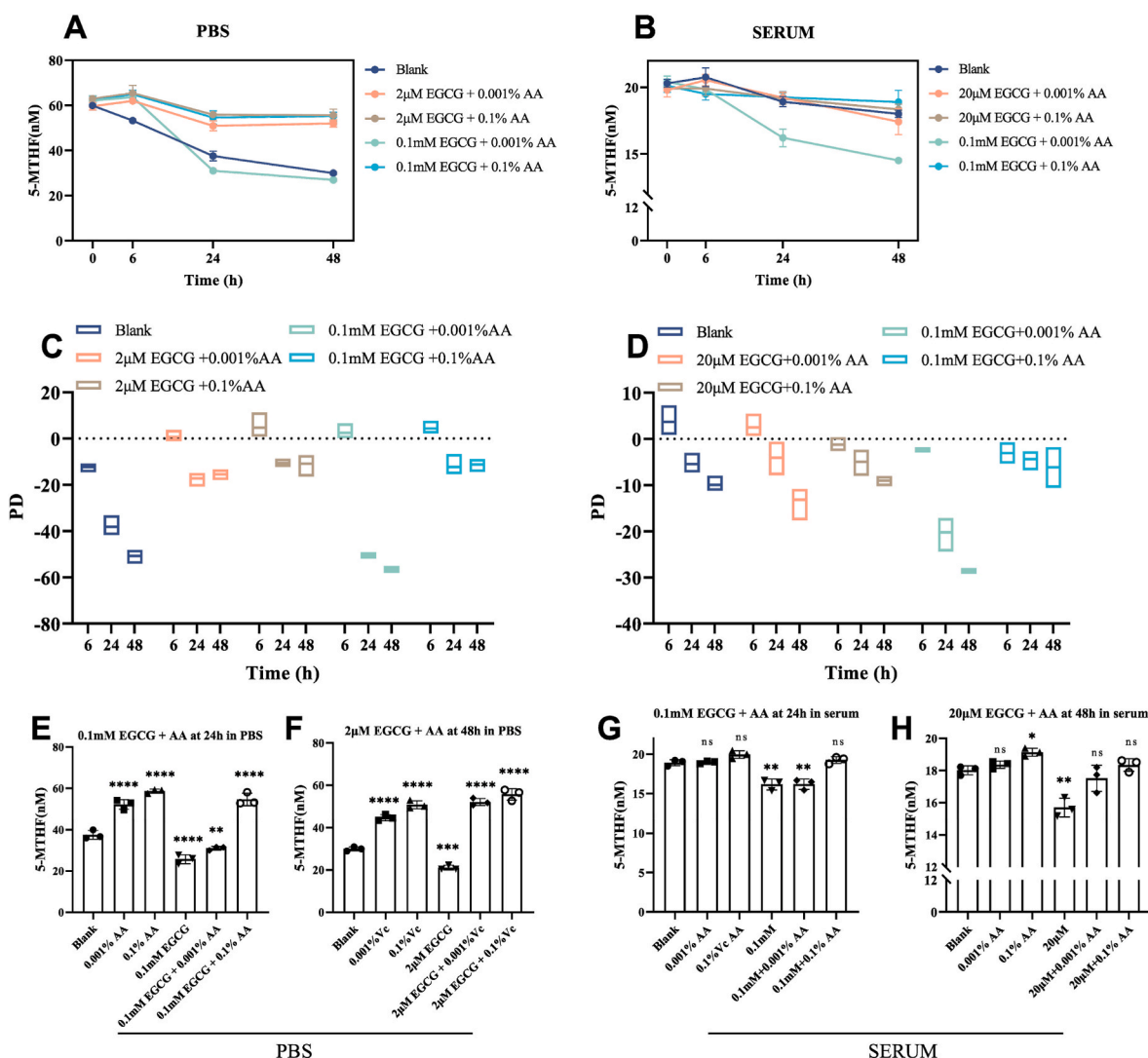
Fig. 2. The effect of EGCG in combination with AA on 5-MTHF in PBS and serum. (A) Effect of EGCG + AA on 5-MTHF in PBS. (B) Effect of EGCG + AA on 5-MTHF in serum. (C) The PD of 5-MTHF treated with EGCG + AA at 6, 24 and 48 h in PBS, Data is represented as median (minimum to maximum). (D) The PD of 5-MTHF treated with EGCG + AA at 6, 24 and 48 h in serum, Data is represented as median (minimum to maximum). (C) Effect of 0.1 mM EGCG + AA on 5-MTHF in PBS at 24 h. (D) Effect of 0.1 mM EGCG + AA on 5-MTHF in serum at 24 h. (E) Effect of 2 μM EGCG + AA on 5-MTHF in PBS at 48 h. (F) Effect of 20 μM EGCG + AA on 5-MTHF in serum at 48 h. Values are mean ± SD, n = 3 per group, $^{ns}p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

MTHF in both the solution and serum, H_2O_2 no longer continued to exert its pro-oxidant effect, suggesting that H_2O_2 had been consumed or degraded.

4. Discussion

We observed that EGCG had an impact on 5-MTHF whether in solution and serum. In the solution, concentrations of EGCG ranging from 2 μM to 1 mM exhibited a pro-degradation effect on 5-MTHF. However, EGCG took longer to show its effects and required higher concentrations in serum compared to the solution. The solution, being a simpler system, made it easier to observe EGCG's influence on folates. In contrast, serum presented a more complex environment, potentially influenced by enzymes, proteins, and other substances. EGCG has the capability to non-covalently bind with human serum albumin (HSA) [29]. The free sulf-hydryl groups on HSA can also act as antioxidants, aiding in EGCG stability. Moreover, Serum contains Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), enzymes that scavenge ROS in the blood, maintaining a lower oxygen pressure in serum, thus contributing to the stability of 5-MTHF. However, the absence of these

antioxidant substances in PBS may lead to a more pronounced pro-degradation effect. Our findings in the solution differ from previous reports. Rozoy et al. [30]found that 400 mg/mL EGCG in a pH 5.5 buffer could protect 1 mM 5-MTHF from oxidative degradation. However, our study differs in EGCG concentration, 5-MTHF concentration, pH, and experimental duration.

EGCG is a polyphenol, both its antioxidant and pro-oxidant proper-ties have been reported. Although these may seem to be contradictory properties, the structure of EGCG is such that both properties exist simultaneously: the antioxidant property of EGCG is derived from the phenolic hydroxyl groups providing protons to scavenge various free radicals; Its pro-oxidant property emerge from the formation of semi-quinone structures during auto-oxidation, leading to the generation of ROS [28]. EGCG is not stable in solution. At low concentrations (μM), EGCG undergoes auto-oxidation, forming semiquinone intermediates, and generating superoxide anions, further producing H_2O_2 and other ROS. Conversely, high concentrations of EGCG (mM) are relatively more stable and tend to undergo epimerization Fig. 4. Therefore, high con-centrations of EGCG result in lower concentrations of H_2O_2 in solution, after the equilibration of EGCG denaturation, high concentration of
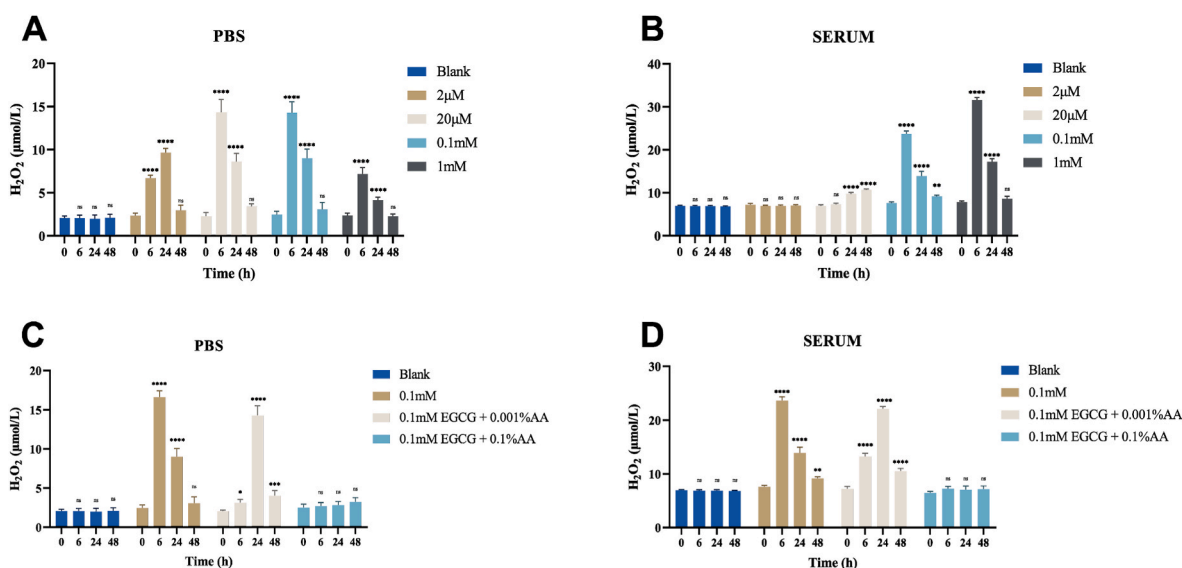
Fig. 3. EGCG induced the degradation of 5-MTHF by generating H_2O_2. (A) Levels of H_2O_2 generated by different concentrations of EGCG in PBS. (B) Levels of H_2O_2 generated by different concentrations of EGCG in serum. (C) Levels of H_2O_2 generated by EGCG + AA in PBS. (D) Levels of H_2O_2 generated by EGCG + AA in serum. Values are mean $\pm$ SD, n = 3 per group, $^{ns}p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.
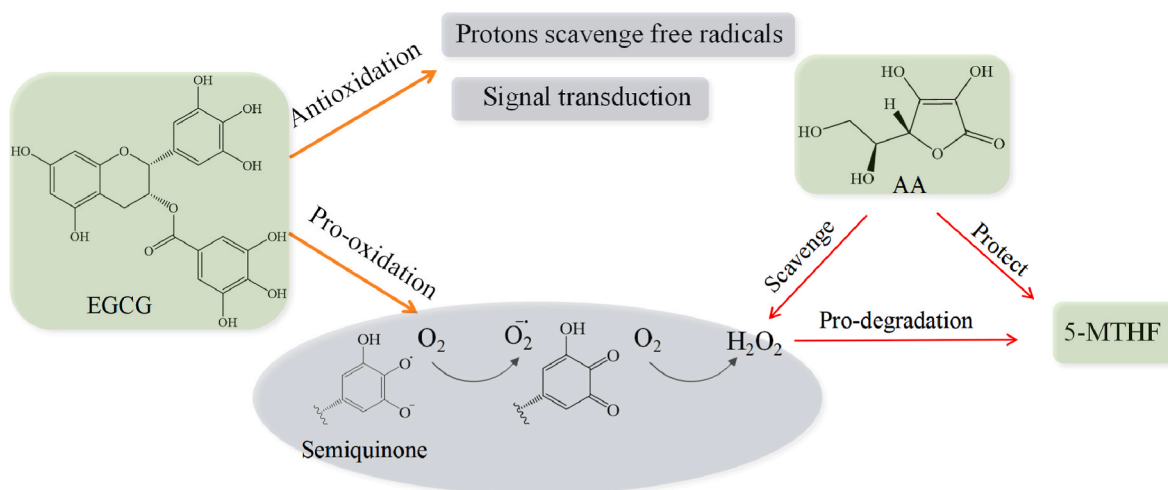

Fig. 4. Mechanism of EGCG affects 5-MTHF.

EGCG (1 mM) can exert antioxidative effects to maintain the level of 5-MTHF in the solution (Fig. 1E and F).

Huennekens et al. [19] and Blair et al. [20] reported that H_2O_2 can induce the degradation of 5-MTHF. Our investigation revealed the presence of H_2O_2 produced by EGCG in both solution and serum (Fig. 3). The role of H_2O_2 also explains the changing trend of PD in different matrices. The impact of EGCG on 5-MTHF contrasts with the direct addition of H_2O_2, likely due to EGCG's gradual degradation and subsequent release of H_2O_2. When we directly added 30 μM H_2O_2 to the solution and serum, it did not significantly alter the levels of 5-MTHF. This is because directly added H_2O_2 is unstable and rapidly degrades. Therefore, when we introduced high concentration of H_2O_2 (2 mM), 5-MTHF underwent partial oxidation and subsequently displayed a trend of natural degradation. Additionally, we proved that AA can eliminate the H_2O_2 generated by EGCG, thereby stabilizing 5-MTHF. One noteworthy point is that AA can prevent the denaturation of 5-MTHF. This suggests that the protective effect of L-ascorbic acid on the degradation of 5-MTHF may not only be attributed to its antioxidative properties but also to other potential mechanisms.

People with a habit of drinking tea tend to intake a considerable amount of EGCG, which could potentially affect the body's folate levels. AA is a powerful antioxidant that scavenges various ROS [31]. To mitigate this risk, we investigated the role of AA. Our results demonstrated that 56 μM AA in the solution effectively counteracted the anti-folate effect of 2 μM EGCG. Similarly, in serum, 56 μM AA was sufficient to reverse the pro-degradation effect of 20 μM EGCG on folate. This suggested that individuals who frequently consume tea can maintain folate levels by timely supplementing with AA. Furthermore, we observed that when the concentration of EGCG surpassed that of AA, AA could not effectively sustain 5-MTHF. This indicated that if EGCG accumulates in the bloodstream at a higher concentration than the body's intrinsic stabilizing capacity, and without the timely addition of exogenous antioxidants, EGCG might influence the body's folate levels. Furthermore, in in vivo studies, co-uptake of EGCG with AA has been shown to enhance the stability of EGCG and increase its bioavailability [32]. A clinical trial indicated that simultaneous intake of 5-MTHF and AA significantly increased the levels of 5-MTHF in the human body [33]. Therefore, it is necessary to avoid continuous intake of large amounts of

green tea and to timely supplement antioxidant substances.

CRediT authorship contribution statement

Guangbin Zhou: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mengmeng Zhang:** Supervision, Resources, Project administration, Conceptualization. **Xiaoyu Sun:** Resources, Funding acquisition. **Ting Huang:** Supervision, Methodology. **Kun Hou:** Writing – review & editing, Supervision. **Siqi Zhou:** Project administration. **Jun Yin:** Supervision, Resources, Project administration, Funding acquisition. **Liping Guan:** Resources, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101719.

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