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Epidermal Growth Factor Stability and Cell Proliferation Enhanced by Antioxidants

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Abstract

Human recombinant epidermal growth factor (EGF) promotes cell proliferation as well as skin remodeling and healing. However, it's unclear if EGF is stable in *in vitro* cell culture medium treatment conditions. Since EGF's instability in aqueous solutions has resulted in different kinds of challenges, novel treatment approaches that preserve EGF's stability and usefulness have been developed. The current study examined the effects of the antioxidants sodium selenite (Se), disodium ethylenediaminetetraacetic acid dihydrate (EDTA), zinc chloride (Zn), and ascorbic acid (AA) on the stability of EGF in cell culture using Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI), as well as their ability to scavenge free radicals. *In vitro*, DMEM and RPMI serum free medium with 2.5 μM of EDTA, Se, AA, and Zn antioxidants were used separately at 37˚C for three days to examine the stability of EGF. The stability of EGF was evaluated using reversed phase high-performance liquid chromatography (RP-HPLC). Cells were cultivated for 3 days to further estimate the impact of antioxidants on cell cytotoxicity and proliferation using the MTT assay with NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cell types. The antioxidants EDTA, Se, AA, and Zn at an extracellular concentration of 2.5 μM improved EGF stability in both DMEM and RPMI medium while having no effect on the proliferation of NIH-3T3, L929, TF-1, and SP2/0-Ag14 cells.

Keywords

Cytotoxicity, Antioxidants, EGF Stability, RP-HPLC Analysis, Peroxide Level

1. Introduction

The natural production of EGF, a short polypeptide hormone, promotes the processes of proliferation, expansion, and division of cells [1]. For *in vitro* cell culture,

EGF functions as a growth factor [2] and has an effective mitogenic effect on endothelial cells, fibroblasts, and most epithelial tissues. Its biological functions rely on associating itself with a specific cell membrane receptor [3]. Because EGF plays a crucial role as a mitogen in the proliferation of various cell types both *in vivo* and *in vitro*, it has been used in the therapeutic and cosmetic areas [4] to cover scars and reduce the appearance of aging skin [1]. Moreover, recombinant EGF is used topically for diabetic foot ulcers [5]. The structures and properties of proteins vary; favorable conditions are necessary for conformation, stability, and proper function. In contrast, a protein degrades, denatures, or precipitates when it is exposed to unfavorable conditions or when its natural environment changes suddenly. Recombinant human EGF is most frequently degraded by oxidation and deamidation [6]. These reactions typically have long-term implications. For protein solutions to remain stable and have a longer shelf life, excipients may need to be added, depending on how the protein is used in the experiment and other factors. When it comes to the chemical and physical degradation of proteins, the solution environment plays a crucial role in protein formulations. Of particular concern are buffer types, pH, and antioxidants [7]. Even though antioxidants assist in stability and solubility in liquid solutions, which help to preserve protein structure and function, they are frequently considered inactive ingredients in pharmaceutical compositions [8] [9].

Since an unstable protein solution can impact the product's appearance, potency, purity, healing effects, and cell proliferation, *in vitro* protein stabilization is an essential practical consideration for the development of an effective EGF formulation. The stability of EGF in solution has been well documented in several *in vivo* solutions [10]. Though there have been numerous reports on EGF stability, none have specifically addressed treatment in cell culture conditions. Since it has a big influence on several aspects of the parenteral formulation creation process and EGF-based cell proliferation, the study of EGF stability in cell culture medium has gotten little attention. But since many of these *in vitro* tests are conducted in non-physiological settings, such as organic solvents or acidic solutions [11], they frequently fail to yield qualitatively positive results in cellular therapies. Investigation of the stability of EGF in aqueous solutions at 37˚C in cell culture media is relevant, since EGF is highly labile in aqueous systems and its half-life decreases substantially at that temperature [11]. Reducing sugars has the most detrimental effect on EGF stability, and it is demonstrated by substances such as PEG 6000 and polysorbates 20 and 80 that oxidation can increase [12]. Likewise, reactive oxygen species (ROS) are considered cytotoxic because of their capacity to oxidize EGF and cause oxidative damage to cellular components [13]. When aerobic cells are cultured *in vitro*, at higher O_2 concentrations than *in vivo*, free radicals are continually created in the cells [14]. High concentrations of free radicals harm cells *in vitro* [15], but *in vivo*, defense mechanisms protect cells from damage [16]. The EGF's stability in DMEM and RPMI medium administered to *in vitro* cells is not known yet. Therefore, it is very important to comprehend

how stable EGF is in cell culture media when antioxidants are present. Because of their exceptional antioxidant and radical-scavenging features, EDTA [17], Se [18], AA [19], and Zn [20] were selected for this study to address the oxidative effect of EGF stability and cell proliferation. These antioxidants are expected to reduce oxidative damage to EGF in the cell culture medium while also promoting cell proliferation.

Analytical techniques for EGF stability conditions and excipients [12], as well as degradation products [21], have been developed using HPLC from a variety of sources, including cosmetic raw materials [22], culture medium [23], culture broth [24], freeze-dried parenteral formulations [25], gastric juice [26], human urine [27] and acid extracts [28]. Additionally, EGF stability secondary structure and aggregation were examined by circular dichroism (CD) and differential scanning calorimetry (DSC) [29]. However, RP-HPLC is increasingly being used to analyze EGF and its breakdown products *in vivo* and *in vitro*. Within this study, DMEM and RPMI without serum medium were used to separately assess the impact of four different antioxidants such as EDTA, Se, AA, and Zn on EGF stability. The EGF samples' stability was examined using RP-HPLC. Furthermore, a biological assessment of EGF with antioxidants was performed *in vitro* utilizing four different cell types.

2. Materials and Methods

2.1. Materials

Human recombinant Epidermal Growth Factor (1 mg/mL of Lyophilized EGF was reconstituted in 1 mL of Milli-Q water, to test the EGF stability and other experiments. DMEM and RPMI medium were used to dilute the stock solution to 50 µg/mL, the unused stock EGF solution was kept at −20˚C and Thermo Fisher Scientific was the supplier of the 0.25% trypsin-EDTA solution (Cranbury, NJ, USA). Millipore Sigma (Burlington, MA, USA) was the source of the Disodium ethylenediaminetetraacetic acid dihydrate, Sodium selenite, L-Ascorbic acid and zinc chloride. A stock solution of disodium ethylenediaminetetraacetic acid dihydrate, Sodium Selenite, Ascorbic acid and Zinc chloride was prepared separately in Milli-Q water. These solutions were utilized as sources of antioxidants for assays including peroxide levels, cell proliferation, EGF stability, and in vitro cytotoxicity. Ferrous (F^{2+}) sulfate heptahydrate, ferric (F^{3+}) chloride hexahydrate, barium chloride dihydrate, and ammonium thiocyanate (750 mg of ammonium thiocyanate were dissolved in 2.5 mL of Milli-Q water, and the solution was then stored at room temperature) are all purchased from Thermo Fisher Scientific (Cranbury, NJ, USA). Fisher Scientific (Cranbury, NJ, USA) provided the acetonitrile for the HPLC, Sigma Aldrich (Burlington, MA, USA) supplied the Trifluoroacetic Acid (HPLC Grade), Ricca (Houston, TX, USA) supplied the 10N hydrochloric acid, Waters (USA) supplied the LectraBond cap preslit PTFE/silicone septa, and Ricca supplied the 12 × 32 mm glass screw neck vial quick thread. The American Type Culture Collection (Manassas, VA) provided the mouse fibroblast

cell line NIH-3T3, mouse fibroblast cell line L929, human erythroblast cell line TF-1, and B lymphocyte cell line Sp2/0-Ag14. Life Technologies Corporation (Grand Island, NY, USA) supplied the Roswell Park Memorial Institute 1640 medium, Dulbecco's Modified Eagle Medium, and Eagle's Minimum Essential Medium (MEM). The Milli-Q water is used to make solutions using in-house MilliQ equipment (Millipore, Milford, MA, USA). Cell culture flasks were obtained from Corning Company (Corning, NY, United States). The remaining chemicals were all analytical grade and obtained from reliable commercial vendors. The use of the DMEM, MEM, and RPMI media of the related cell type being treated, each of the stock solutions and reagents had been prepared fresh prior to the assay, and dilutions were made to the appropriate concentrations.

2.2. Cell Type and Cell Culture Conditions

NIH-3T3 (mouse fibroblast) and Sp2/0-Ag14 (mouse B lymphocyte) cells were cultured in DMEM supplemented with 0.01 mg/ml streptomycin sulfate, 100 U/ml penicillin, and 10% fetal bovine serum (FBS). MEM containing 10% FBS, 100 U/ml penicillin, and 0.01 mg/ml streptomycin sulfate (complete medium) was used to cultivate L929 (mouse fibroblast) cells. Because L929 cells are adherent, the sub-culturing step was carried out first. Following the removal of the old media, the cells were washed out with approximately 10 mL of PBS (pH 7.4). After that, 2 - 3 mL of 0.25% Trypsin-EDTA solution in Hanks balanced salt solution without calcium or magnesium was introduced, after which the cells were allowed to detach/dissociate from their monolayers for two to three minutes at room temperature. Following their dilution in a complete medium, the cells were counted. After seeding the cells at a density of 2500 cells/well in triplicate wells of 96-well plates, they were left to adhere overnight before the cytotoxicity and proliferation experiments were carried out. Following that, the medium was swapped out for new, complete medium before antioxidants and EGF were added. Human erythroblast (TF-1) cells were cultured in RPMI 1640 supplemented with 0.01 mg/ml streptomycin sulfate, 10% FBS, and 100 U/ml penicillin (complete medium). All cells were kept in an incubator that was humidified at 37˚C with 5% CO_2, and the medium was changed every two to three days with fresh, complete medium. A cell suspension's viable cell count was determined using the dye exclusion method [30]. Throughout the investigation, all cells were kept at about 90 percent viability.

2.3. Analyzing the Cytotoxicity with Different Antioxidants

The cytotoxicity of four independent antioxidants such as EDTA, Se, AA, and Zn on four different NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cell types had been evaluated using 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The MTT test, which is based on the tetrazolium is reduced metabolically to produce a color (formazan) product by mitochondrial succinate dehydrogenase, shows a linear connection between cells that are metabolically active and the

intensity of the color of the purple formazan solution. This can be quantified using spectrophotometric measurements at 570 nm. After the data have been collected, the change in cell proliferation or apoptosis is calculated. Individually, 50 µL of NIH-3T3, L929, TF-1, and Sp2/0-Ag14 Cell suspensions were seeded in 96-well microculture plates at a density of 2500 cells/well. Then, 50 µL of full medium containing varying concentrations of antioxidants (0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, 20, & 40 µM) of EDTA, Se, AA, and Zn was added to each cell well independently.

The control studies for each cell type were carried out independently using a complete medium devoid of antioxidants, and the complete medium was used as a reagent blank. To evaluate cytotoxicity, the culture plates were cultured for three days at 37˚C and 5% CO_2 in a humidity-controlled incubator. Following three days of exposure, 100 µL of a solution of MTT (5 mg/mL in PBS, pH 7.4) was added to each well, including the blank and control wells. The plates were then incubated in an incubator with 5% CO_2 at 37˚C. Four hours later, each cell well received 100 µL of a solubilization combo solution (10% sodium dodecyl sulfate and 0.01 N hydrochloric acid). After that, the plate was put in a humidified incubator set at 37˚C with 5% CO_2 to dissolve the formazan crystals overnight. The absorbance of the resultant color solution was measured at 570 nm. The medium's background absorbance was evaluated in wells containing complete medium and MTT solution that did not include cells. The calculated cytotoxicity is represented as a percentage of cell viability using below Equation (1).

$$\text{Cell Viability}(\%) = \frac{\text{Optical density of test sample} - \text{Optical density of Blank}}{\text{Optical density of Control} - \text{Optical density of Blank}} \times 100 \quad (1)$$

The above equation describes the measured absorptions for the test, control and blank optical densities. The calculated cytotoxicity is shown as a percentage of the survival of cells. Using the average percentage from three different experiments, the results were displayed.

2.4. Proliferation of Different Cells with EGF and Different Antioxidants

The EGF biological activity with different antioxidants was assessed using an MTT test with four different NIH-3T325, L929, TF-1, and Sp2/0-Ag14 cell types. This assay is based on viable cells' mitochondrial dehydrogenase reducing the tetrazolium salt MTT to form a purple insoluble formazan product. Separately, 50 µL of NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cells were placed in 96-well microculture plates at a cell density of 2500 cells/well. Then, 50 µL of EGF in varying amounts (0.019, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5 & 10 ng/mL) with 2.5 µM of antioxidants, including EDTA, Se, AA, and Zn in complete medium, were added one at a time. Control experiments were carried out independently for each cell type using the complete medium: negative control cells grew in the complete medium without the addition of EGF or antioxidants, while positive control cells grew in the complete medium with 2 ng/mL of EGF but no antioxidants. The

complete medium was also used as a reagent blank.

For three days, the cells in the plates were grown at 37˚C in an incubator with 5% CO_2 and humidity. After that, 100 µL of the MTT solution was applied to all wells, including the blank and control wells. The plates continued to incubate at 37˚C with 5% CO_2 in a humidified incubator.

The formazan crystals were dissolved by adding 100 µL of a solubilization solution to each well after a 4-hour incubation period. Subsequently, the plates were then incubated with 5% CO_2 at 37˚C overnight. A wavelength of 570 nm was used to measure the absorbance of the resulting solution. In wells containing a complete medium, the background absorbance of the medium was measured. In relation to the positive control results, the percentage of cell proliferation at each EGF amount with different antioxidants was reported. The calculated cell proliferation is represented as a percentage of cell growth using **Equation (1)**. Three separate experiments were carried out.

2.5. Reverse Phase-HPLC Conditions

An Agilent 1100 series apparatus, consisting of a variable wavelength detector, thermostat column compartment, online degasser, auto sample injector, injecting loop, and quaternary pump solvent supply module, was used to perform the RP-HPLC. Prior to analysis, the EGF sample vials were kept at 5˚C in the self-sampling chamber. The instrument operated at room temperature (20 ± 2˚C) in an HPLC lab. The analysis was conducted on a C18, Vydac 218TP (250 × 4.6 mm), 5 µm column with a steady 30˚C column temperature. In mobile phase A included 0.1% trifluoroacetic acid (TFA) in water, while mobile phase B had 0.1% TFA in acetonitrile for 0 - 15 minutes, the EGF was separated using 10 - 40% B gradient elution. The EGF peak absorbance was measured using a 200 nm wavelength, a 1 mL/min mobile phase flow rate, and a 10 µL sample injection volume. Empower software version 3 was used to obtain chromatogram output, peak integration, peak area computation, and retention times.

2.6. Stability of EGF Analyzed in DMEM and RPMI Medium with Antioxidants

To test the influence of four different antioxidants such as EDTA, Se, AA, and Zn on EGF stability, following the EGF stock solution has been prepared to 50 µg/mL in DMEM and RPMI mediums, 2.5 µM of each antioxidant was added one at a time. Two separate control vials were prepared with 50 ng/mL of EGF from each DMEM and RPM medium, but no antioxidants were contained. Following that, at 37˚C each testing and control vials were incubated for three days, while the second control vial from each medium was incubated at 2 - 8˚C for three days. Three days later, each sample solution was analyzed independently using RP-HPLC, and the EGF stability results were compared to freshly made EGF from stock and control. The percentage of EGF recovered from the RPMI and DMEM medium was then determined using empower soft-

ware version 3.

2.7. Examine the Peroxide Level in RPMI and DMEM Media When Antioxidants Are Present

2.7.1. Ferrous(II) Chloride Solution Preparation

In 49 milliliters of Milli-Q water, 0.5 grams of ferrous(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) were dissolved to create a solution of ferrous(II) chloride. Barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) weighing around 0.4 grams was dissolved in 49 milliliters of Milli-Q water. The ferrous(II) sulfate heptahydrate solution was gradually combined with a barium chloride solution. Following a thorough mixing process, 2 mL of 10 N HCl was added. Following thorough stirring, let the barium sulfate precipitate for 20 to 30 minutes at room temperature (RT). Using a 0.45 Micron filter, the barium sulfate precipitate was removed to provide a clear ferrous(II) chloride ($FeCl_2$) solution. The resulting solution was then stored in a brown bottle at RT.

2.7.2. Standard Ferric(III) Chloride Solution Preparation

To make standard ferric(III) chloride ($FeCl_3$), 0.4 gram of ferric(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was dissolved in 49 mL of Milli-Q water, followed by the addition of 1 mL of 10 N HCl. After thoroughly mixing the mixture, it was kept at RT in a brown bottle.

2.8. Determined Peroxide Level in DMEM and RPMI Medium with Antioxidants

Peroxide levels in DMEM and RPMI medium were determined using a modified International Dairy Federation (IDF) approach [31]. To test for peroxide levels, dilute the stock EGF solution to 50 μg/mL in DMEM and RPMI medium. subsequently, 2.5 μM of EDTA, Se, AA, and Zn were added individually. The control sample made 50 μg/mL EGF in DMEM and RPMI medium without antioxidants. The sample and control vials were covered with aluminum foil and incubated for three days at 37˚C to protect the reaction mixture from light. After incubating at 37˚C for three days, 3 μL of ferrous(II) chloride (15 μg/mL final) and 15 μL of ammonium thiocyanate solutions were added, and the mixture was thoroughly mixed using a vortex. To get optimum color development, the sample tubes are heated in a water bath at 50˚C for 2 minutes. To bring the tubes to RT, they were kept on ice for two minutes [32]. After that, mix well and leave all the tubes in a dark place for five minutes at RT, following that, transfer 200 μL into 96-well plate from each sample tube. The test sample and control were immediately measured for color absorbance at 505 nm using a spectrophotometer. The test outcomes were then compared with the controls, which were DMEM and RPMI mediums free of antioxidants.

3. Results

The safeguarding effect of different antioxidants on the stability of EGF in RPMI

and DMEM medium were investigated.

3.1. Cells Cytotoxicity Depends on Cell Type and Antioxidant Concentration

The cytotoxicity of four different NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cell types was studied by treating them independently with varying concentrations of antioxidants EDTA, Se, AA, and Zn, ranging from 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, 20, and 40 μM. The control cells were cultivated in complete medium devoid of antioxidants, while the reagent blank was a complete medium. Cell viability was assessed over a three-day period using a colorimetric method based on MTT. Following three days of exposure to various antioxidants, the cytotoxicity results for various cells are shown in **Figure 1(A)-(D)**. After 3 days of exposure to EDTA concentrations ranging from 0.078 to 40 μM, NIH-3T3 and Sp2/0-Ag14 cells showed substantially improved viability. Although the cells' vitality was higher than the comparable controls, this shows that NIH-3T3 and Sp2/0-Ag14 cells are more resistant to the maximum concentration of EDTA that was examined (**Figure 1(A)** and **Figure 1(D)**). Given that both TF-1 and L929 cells show EDTA sensitivity at higher tested doses, it is possible that these fibroblasts are more susceptible to EDTA at greater concentrations [33]. L929 cells' viability was decreased by EDTA at 20 μM or higher, but TF-1 cells were more vulnerable at 40 μM or higher (**Figure 1(B)-(C)**). This implies that EDTA's effects on cells *in vitro* differ based on the type of cell and the concentration used [17]. Similarly, NIH-3T3 cells are resistant to Se concentrations up to 5 μM, while L929, SP2/0-Ag14, and TF-1 cells are resistant up to 10 μM and 20 μM, respectively. However, at higher concentrations, all four cells exhibited Se sensitivity (**Figure 1(A)-(D)**). The studies suggest that Se's biological effects on cell viability are concentration-dependent [34]. Although findings indicate that Se stimulates cell proliferation at low concentrations, higher concentrations of Se may increase the generation of ROS and induce oxidative stress [35]. However, higher Se concentrations probably caused oxidative stress on all four cell types in the medium, which lowers viability, in contrast to the corresponding control.

How AA affects cytotoxicity was examined at various concentrations, and at or above 5 μM was found to reduce the NIH-3T3 cell viability, which decreased as the AA concentration increased. At 40 μM of AA, TF-1 cells' viability was significantly reduced. However, at lower concentrations, compared to the control cells, the TF-1 cells were not susceptible to AA and had no effect on cell survival. Similarly, L929 and Sp2/0-Ag14 cells treated with varying concentrations of AA showed increased cell viability and did not reduce cell viability up to 40 μM compared to their control cells, indicating that AA may promote L929 [36] and Sp2/0-Ag14 cells growth. **Figure 1(A)-(D)** shows the cytotoxicity effect of AA on NIH-3T3, L929, TF-1 and Sp2/0-Ag14 cells. The cytotoxicity evaluation of NIH-3T3 cells at increasing Zn concentrations indicates a decrease in cell viability at or above 20 μM. Similar concentrations of Zn with L929 cells did not affect cell via-

bility up to 40 μM and viability of L929 cells more than that of control group. Zn treated with TF-1 and Sp2/0-Ag14 cells reduced their viability at or above 5 μM, with an increase in Zn concentration, cell viability decreased. After 3 days of treatment, indicate that Zn cytotoxicity is dependent on cell types [37] and has a sensitive concentration dependent response cytotoxic effect [38] (**Figure 1(A)-(D)**).

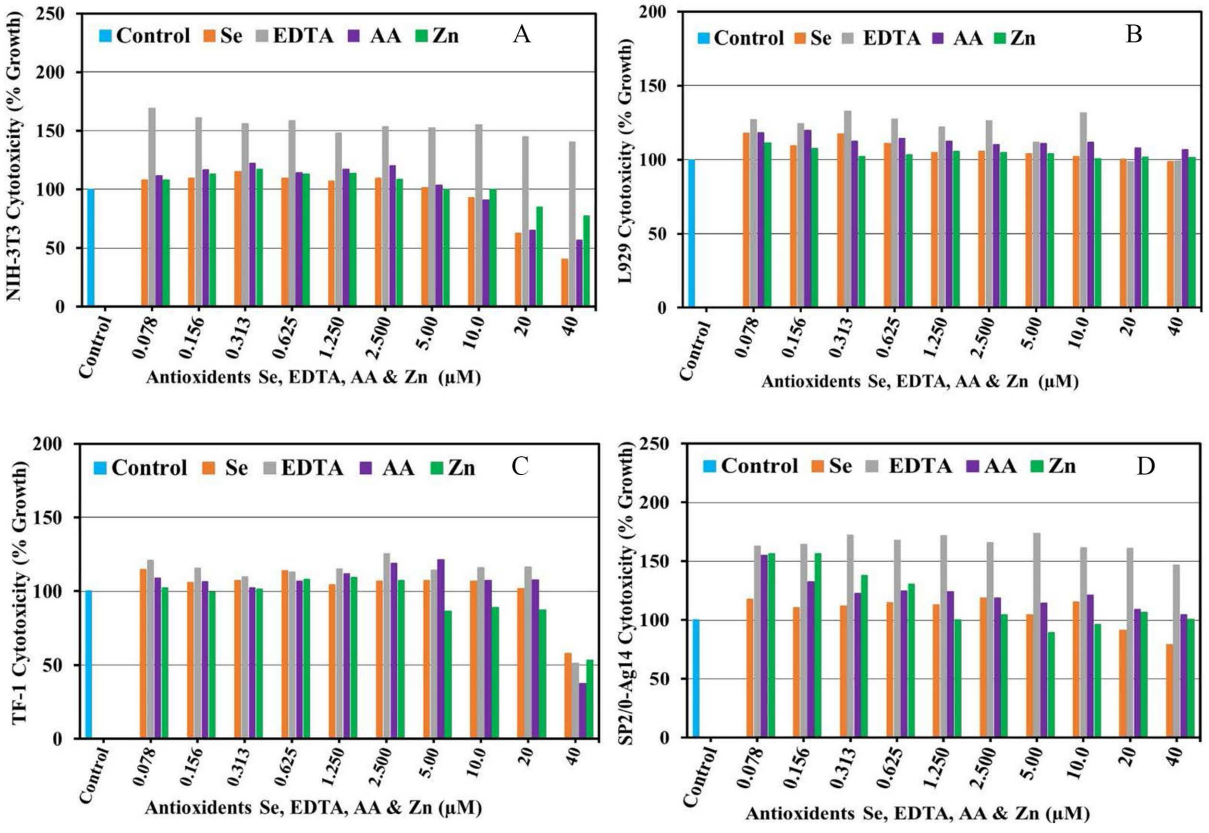

Figure 1. Four different NIH-3T3 (A), L929 (B), TF-1 (C), and Sp2/0-Ag14 (D) cells were treated separately with varying amounts of the antioxidants EDTA, Se, AA, and Zn, ranging from 0.078 to 40 μM, to assess their cytotoxicity. The MTT-based colorimetric assessment was employed to estimate cell viability, and the results were evaluated over three days. The reagent blank was complete medium, while the control group was cells grown in complete medium that did not contain antioxidants. The calculated cytotoxicity is shown as a percentage of cell viability. The results were presented as a mean percentage from three separate experiments.

According to the results, each of the four cell types had demonstrated a certain level of sensitivity to each of the four distinct antioxidants. The conclusion that EDTA, Se, AA, and Zn were used to make the EGF solution, demonstrating that this method was safe to use to treat cells in DMEM and RPMI medium, is supported by the intriguing differences in their sensitivities, which imply that the mechanism may be affected by factors built-in to the cellular or biomolecular profiles of each cell type. Although an extracellular concentration of 2.5 μM of EDTA, SE, AA, and Zn applied separately to each of the four cell types did not affect the viability of NIH-3T3, L929, TF-1, and SP2/0-Ag14 cells, making them more biocompatible. In addition, all cell types were more viable than the control group,

which was consistent with the cytotoxicity findings. So, 2.5 μM of EDTA, Se, AA, and Zn were used separately in later studies.

3.2. Antioxidants with EGF Induced Cell Proliferation

The present investigation aims to ascertain whether EGF may promote the proliferation of NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cells in the presence of 2.5 μM EDTA, Se, AA, and Zn separately. The MTT test uses spectrophotometric measurements at 570 nm to linearly identify metabolically active cells based on the measured intensity of color of the purple formazan solution.

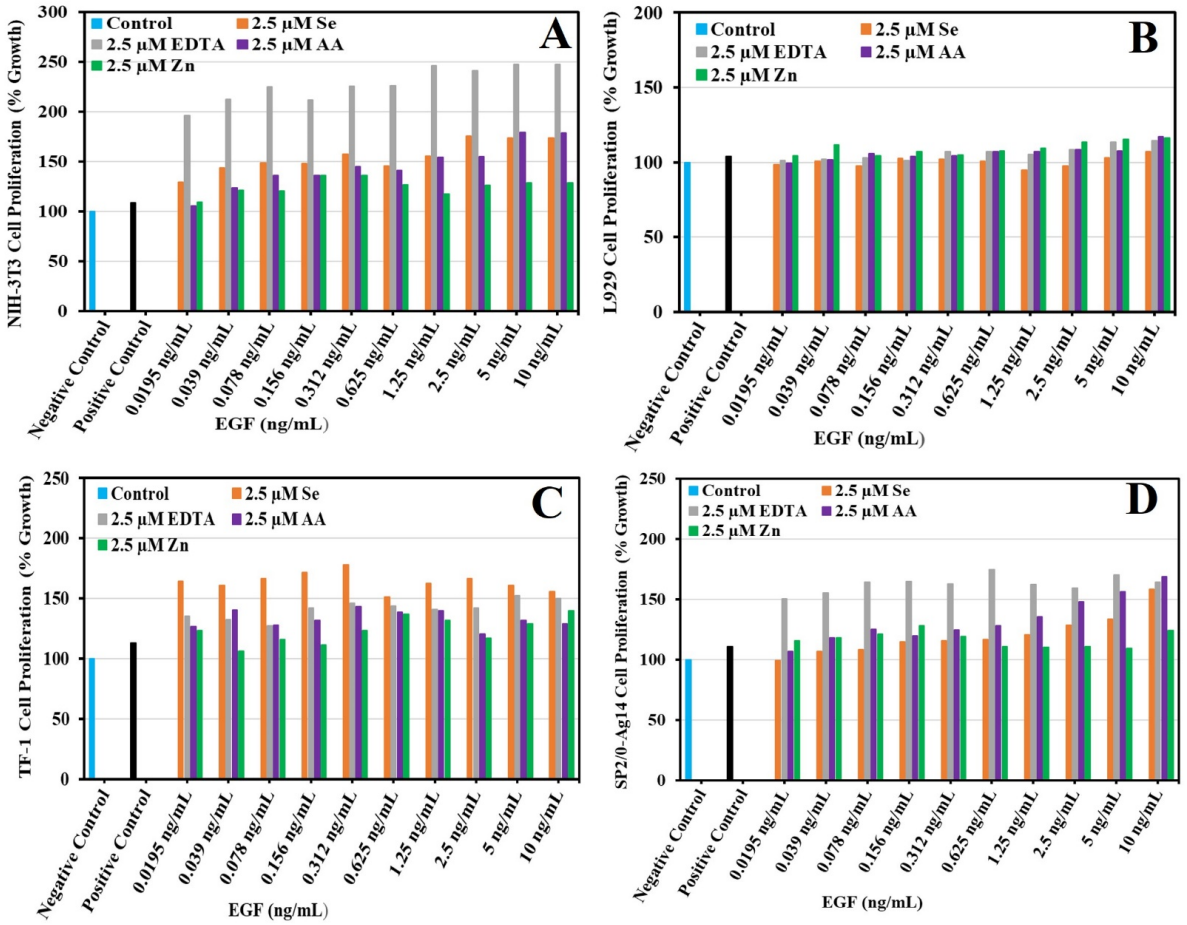

Figure 2. The proliferation of four different NIH-3T3 (A), L929 (B), TF-1 (C), and Sp2/0-Ag14 (D) cells were treated independently with varying doses of EGF (0.019, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 ng/mL) with 2.5 μM of EDTA, Se, AA, and Zn. Control studies were carried out on each cell type using the complete medium individually. The complete medium was used to grow positive control cells with 1 ng/mL of EGF and no antioxidants, in comparison, negative control cells proliferated in a complete media without of antioxidants and EGF and employed the culture medium as a reagent blank. In comparison to the positive control results, the percentage of cell proliferation at each EGF concentration utilizing different antioxidants was reported. The results were presented using the mean percentage from three separate experiments.

Following that, the obtained data is used to determine the change in growth of cells or death. For cell proliferation tests, noncytotoxic antioxidant concentration

of 2.5 μM were chosen to minimize cytotoxicity over the course of a 3-day incubation time. Four different NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cells were treated separately with varying amounts of EGF (0.019, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 ng/mL) with 2.5 μM of EDTA, Se, AA, and Zn. Separate control tests were conducted on every type of cell. While negative control cells grew in the complete medium without EGF and antioxidants, and the culture medium served as a reagent blank, positive control cells were grown in the complete medium with 1 ng/mL of EGF and without antioxidants. After that, each cell was cultured separately at 37˚C with 5% CO_2 for three days in a humidified incubator. The results of different cell proliferations with differing amounts of EGF and antioxidants are presented in **Figure 2(A)-(D)**. The results demonstrate that when 2.5 μM EDTA, Se, AA, and Zn were added separately, the proliferation of NIH-3T3, TF-1, and Sp2/0-Ag14 cells increased with increasing EGF amount when compared to the positive control.

While NIH-3T3 cells needed EGF [39] as a growth factor to proliferate *in vitro*, the addition of EDTA to EGF dramatically increases the proliferation of the cells in comparison to a positive control. When treated independently with varying amounts of EGF and 2.5 μM antioxidants, both Sp2/0-Ag14 and TF-1 cells improved their mitogenic activity. In summary, For TF-1 cells to proliferate, GM-CSF or IL-3 is necessary [40], but Sp2/0-Ag14 and TF-1 cells are thought to be able to grow and proliferate *in vitro* without the need for extra EGF to be added to the medium. The results show that adding antioxidants along with EGF increased the proliferation of these cells in comparison to the corresponding positive control, suggesting that the antioxidants' presence lowers oxidative stress in the medium. Antioxidants such as EDTA, Se, AA, or Zn added separately to EGF did not increase the proliferation of L929 cells; it is believed that L929 cells can grow and proliferate *in vitro* without the need for EGF, but EGF can still affect L929 cell proliferation [41] at higher EGF amounts (5 ng/mL and up) with all antioxidants added separately. However, the cell viability was nearly identical to that of the positive control. Moreover, ROS could control apoptotic procedures in addition to cellular proliferation, survival, and differentiation [42]. This implies that there may be an unfavorable balance in MEM medium between the free radical's generation and antioxidant defenses, as a result oxidative stress may rise [43]. The proliferation of L929 cells, however, may be inhibited by increased oxidative stress in cell culture.

3.3. Analytical Conditions and DMEM and RPMI Medium Components, Did Not Interfere with the Separation of EGF

Stability of EGF in cell culture medium such as DMEM and RPMI was evaluated by analytical RP-HPLC settings as previously reported [44]. The effects of certain column kinds, mobile phases, and detecting equipment configurations were also investigated. The peak was enhanced during the analysis using a volume of injection of 10 μL and a rate of flow of 1 mL/min. As a result, the EGF peak generated by the detection wavelength of 200 nm was found to be highly sensitive and re-

peatable at a retention time of 9.2 minutes, eluting as a single peak. It was verified that, by keeping the column temperature at 30˚C, all the components in the sample solution had been separated adequately in 25 minutes. To test media component interference with EGF peak, 10 µL of each DMEM and RPMI medium was analyzed separately without EGF on the RP-HPLC device (Figure 3 (A) and Figure 3 (C)). The findings indicate that the EGF peak is pure because there were no co-eluting peaks at the EGF time of retention (Figure 3 (B) and Figure 3 (D)).

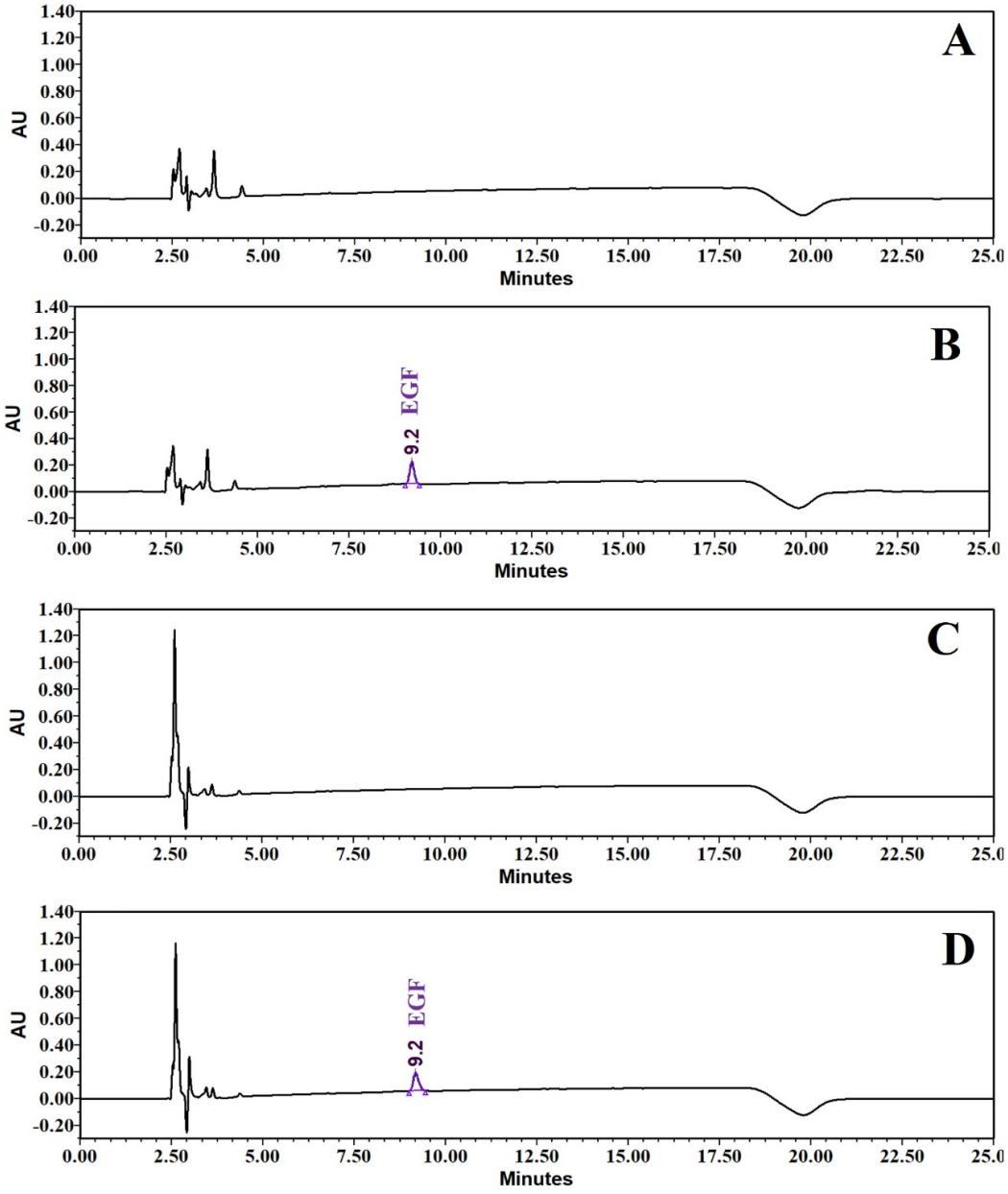

Figure 3. The EGF assessment was unaffected by the DMEM and RPMI media matrices. Ten microliters of each aqueous solution were injected into the HPLC apparatus. DMEM (A), EGF in DMEM (B), RPMI (C), and EGF in RPMI (D). The chromatograms demonstrate the purity of the analyte peak and the absence of any co-eluting peaks that might interfere with the EGF peak's time of retention.

Furthermore, an analytical technique is considered linear if it produces test findings that, within a certain range, are exactly proportionate to the analyte concentration. EGF was examined for linearity in five different concentrations (0.01, 0.02, 0.04, 0.075 and 0.15 μg/μL). The calibration curve was created by charting the peak area against the suitable EGF dosage, and a linear regression analysis was employed to determine the linearity. The EGF standard curve had a coefficient of regression (R^2) higher than 0.99 and was linear over the range. This suggests that there is a significant linear correlation between all the amounts used.

3.4. EGF's Stability in DMEM and RPMI Media Was Enhanced by Antioxidants

The primary EGF breakdown pathways are succinimide production, deamidation, and oxidation, which vary based on the solution's conditions [6]. Antioxidants can stop protein oxidation because they scavenge ROS and balance their production and neutralization [45]. In aqueous solution, EGF has demonstrated remarkable resistance to degradation when shaking stress is applied [29]; EGF stability, however, can be significantly impacted by temperature and other DMEM and RPMI medium ingredients, leading to reduced stability and lack of effectiveness as a treatment. While the solution environment affects the chemical and physical degradation of proteins [7], antioxidants are particularly important. Therefore, to improve EGF stability and reduce the rise in oxidized product or any other likely oxidized types of EGF, this study will examine the effects of antioxidants and radical-scavenging properties of EDTA, Se, AA, and Zn on EGF stability in DMEM and RPMI *in vitro* serum-free medium. Separately, 2.5 μM of EDTA, Se, AA, and Zn were added to 50 μg/mL of EGF solution in serum-free DMEM and RPMI media. After that, all vials containing EGF stability samples were incubated for three days at 37˚C. Each stability sample was determined using RP-HPLC after incubation. The results were compared to freshly generated EGF samples and controls. The study found that the addition of antioxidants EDTA, Se, AA, and Zn at a concentration of 2.5 μM enhanced the stability of EGF in DMEM and RPMI media, preventing oxidation and increasing stability. The EGF stability test results in DMEM and RPMI medium with EDTA, Se, AA, and Zn added separately can be found in **Table 1** and **Table 2**.

Analytical results show that 2.5 μM of each antioxidant independently preserves EGF stability *in vitro* in DMEM (**Table 1**) and RPMI (**Table 2**) medium as compared to controls. Additional investigation will be necessary to fully comprehend the dependent on concentration immediate and long-term impacts of antioxidants like EDTA, Se, AA, and Zn on EGF stability in medium DMEM and RPMI. Antioxidants are chelating and free radical scavenging agents whose primary role in treatment is to prevent metal-induced oxidation of -SH groups and help maintain reduced protein states. These antioxidants could have been used in our study to collect metal ion traces in aqueous solutions because they would have accelerated the oxidation reactions [46]. The study conducted with these antioxidants

demonstrated that it is possible to successfully prevent oxidation processes in EGF aqueous solutions. To further understand the peroxide levels, they were measured in both DMEM and RPMI mediums to assess the effects of each antioxidant.

Table 1. Antioxidants improved EGF stability in DMEM medium. In separate vials, 50 ng/mL EGF solution was prepared in DMEM medium with 2.5 µM of EDAT, Se, AA, and Zn. The vials of samples were incubated for three days at 37˚C. The solutions underwent independent RP-HPLC analysis, and the Empower program, version 3, was utilized to compute the EGF recovery percentage. Results of the experiment are based on the average of three distinct investigations.

	% EGF Recovered		
EGF Samples	**0 Day**	**3 Days at 2 - 8˚C**	**3 Days at 37˚C**
EGF in DMEM freshly prepared	100.0	-	-
EGF in DMEM at 2 - 8˚C without antioxidants	-	95.5	-
EGF in DMEM without antioxidants	-	-	75.2
EGF in DMEM + 2.5 µM Se	-	-	92.4
EGF in DMEM + 2.5 µM EDTA	-	-	96.3
EGF in DMEM + 2.5 µM AA	-	-	93.5
EGF in DMEM + 2.5 µM Zn	-	-	91.7

Table 2. Antioxidants improved EGF stability in RPMI medium. In separate vials, 50 ng/mL EGF solution was prepared in RPMI medium with 2.5 µM of EDAT, Se, AA, and Zn. Vials containing samples were incubated for three days at 37˚C. The solutions were examined independently using RP-HPLC, and Empower software, version 3, was used to determine the EGF recovery percentage. Results of the experiment are based on the average of three distinct investigations.

	% EGF Recovered		
EGF Samples	**0 Day**	**3 Days at 2 - 8˚C**	**3 Days at 37˚C**
EGF in RPMI freshly prepared	100.0	-	-
EGF in RPMI at 2 - 8˚C without antioxidants	-	95.6	-
EGF in RPMI without antioxidants	-	-	81.5
EGF in RPMI + 2.5 µM Se	-	-	95.6
EGF in RPMI + 2.5 µM EDTA	-	-	92.8
EGF in RPMI + 2.5 µM AA	-	-	95.9
EGF in RPMI + 2.5 µM Zn	-	-	96.6

3.5. Determined the Peroxide Value by Analyzing the Linearity

As described in the experimental section, ferric(III) chloride standard solution in water was produced using the ferric(III) chloride stock solution to generate the Fe3+ concentration vs absorbance standard curve. Ferric iron's linearity was examined at different amounts (3, 5, 8, 12, 15, and 20 µg/mL) [32]. The ferric iron concentrations were plotted against the absorbance determined by the plate

reader at 505 nm to create the calibration graph. It was found that the ferric iron coefficient of regression (R^2) was 0.998 and that the standard curve was linear across the whole range (**Figure 4**). The peroxide level, which is measured as milliequivalents of peroxide per kilogram, was obtained by using below **Equation (2)**. The division factor of 2 is used to present the level of peroxide in milliequivalents of peroxide rather than milliequivalents of oxygen.

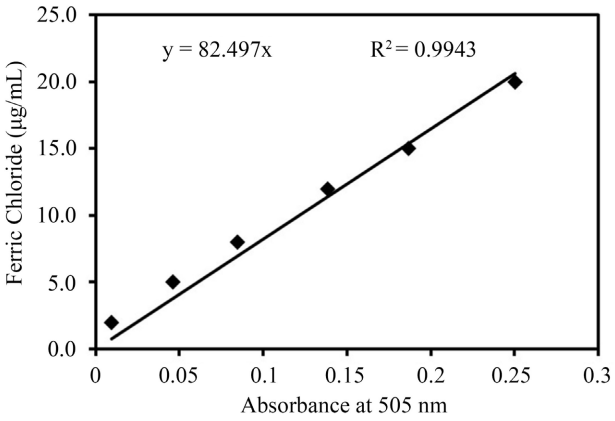

Figure 4. Calibration curve for peroxide level measurement.

$$\text{Peroxide Value}\left(\text{meq/kg}\right) = \frac{\text{Sample absorbance} - \text{Blank absorbance} \times \text{S}}{55.84 \times \text{W} \times 2} \quad (2)$$

S = Standard curve slope.

W = Weight of sample (in grams).

55.84 = Iron's atomic weight.

3.6. Antioxidants Declined the Peroxide Level in DMEM and RPMI Medium

Every laboratory reagent, solution, and medium used for the growth of cells, including DMEM and RPMI [47], is known for containing a variety of transition metal ions, such as copper, iron and nitrate with potent pro-oxidant qualities [48]. However, among the kinds of oxidative stress-induced protein oxidation that are therapeutically relevant is metal-catalyzed oxidation (MCO) [49]. Consequently, it can be a serious issue that EGF, especially at very low concentrations in serum-free medium and other formulations, is often vulnerable to oxidative degradation. A class of compounds known as antioxidants can stop oxidation happening [48], although cell culture media normally lacks them. Finding out if the antioxidant and radical-scavenger properties of EDTA, Se, AA, and Zn would cause the peroxide levels in the DMEM and RPMI medium to reduce was the goal of this study. As explained in the section on experiments, 50 µg/mL of EGF was incubated independently in DMEM and RPMI medium with 2.5 µM antioxidants (EDTA, Se, AA, and Zn) at 37˚C for three days. Following that, the value of peroxide was determined using the IDF method, a spectrophotometric method that measures

the peroxide induced conversion of ferrous ions to ferric ions. The results showed that all four antioxidants EDTA, Se, AA, and Zn declined the peroxide level in both DMEM (**Figure 5(A)** and **Table 3**) and RPMI (**Figure 5(B)** and **Table 4**) medium when compared to the control. It was shown that the antioxidants EDTA, Se, AA, and Zn might potentially lessen the effects of solution-induced protein oxidation caused by peroxide [50]. Furthermore, these antioxidants are probably going to interact with free radicals and metal ions within DMEM and RPMI medium to prevent the reaction before it impacts EGF.

Table 3. Antioxidants decreased the peroxide level in DMEM medium. The amount of peroxide in serum-free DMEM media has been investigated in relation to the antioxidants EDTA, Se, AA, and Zn. In DMEM medium, a 50 ng/mL EGF solution was prepared using 2.5 μM of EDTA, Se, AA, and Zn separately then incubated for three days at 37˚C. The amount of peroxide was then measured in milliequivalents per kilogram. In DMEM medium, the antioxidants EDTA, Se, AA, and Zn decreased the peroxide level in comparison to the control. The average of three independent experiments yields the result.

Sample	Peroxide Value (meq/kg)
EGF in DMEM without antioxidants	0.0158
EGF in DMEM + 2.5 μM Se	0.0103
EGF in DMEM + 2.5 μM EDTA	0.0099
EGF in DMEM + 2.5 μM AA	0.0098
EGF in DMEM + 2.5 μM ZnCl2	0.0110

Table 4. Antioxidants decreased the peroxide level in RPMI medium. The amount of peroxide in the serum-free medium of RPMI was examined in relation to the antioxidants EDTA, Se, AA, and Zn. In RPMI medium, a 50 ng/mL EGF solution was prepared using 2.5 μM of EDTA, Se, AA, and Zn separately followed by three days of incubation at 37˚C. The milliequivalents per kilogram of peroxide were then calculated. In RPMI medium, the antioxidants EDTA, Se, AA, and Zn decreased the peroxide level in comparison to the control. The average of three independent experiments yields the result.

Sample	Peroxide Value (meq/kg)
EGF in RPMI without antioxidants	0.0140
EGF in RPMI + 2.5 μM Se	0.0100
EGF in RPMI + 2.5 μM EDTA	0.0095
EGF in RPMI + 2.5 μM AA	0.0102
EGF in RPMI + 2.5 μM $ZnCl_2$	0.0104

Additional research is required to understand the mechanisms behind the dependent on concentration both immediate and long-term impacts of the antioxidants on the reduction of peroxide content in DMEM and RPMI medium.

4. Discussion

Growth factors are signaling proteins found in cells that have an impact on cell

growth, differentiation, and survival. Proteins must be stabilized *in vitro* to provide an effective formulation because protein formulation that is unstable could damage the product's potency, appearance and purity. It is unclear how stable EGF is when it is employed as a growth factor in media for cell culture at 37˚C because, although its stability in solution [10] was analyzed, little is known about how stable it is in DMEM and RPMI medium.

Because creating stable parenteral formulations depends heavily on identifying a variable solution component that aids in EGF stabilization. For these purposes, four different antioxidants (EDTA, SE, AA, and Zn) that are frequently utilized as metal chelators and free radical species scavengers were assessed and compared for their cytotoxicity, proliferation, EGF stability, and peroxide levels. These antioxidants can slow or prevent cellular damage by scavenging free radicals [51], reducing oxidative damage to EGF, and increasing its stability.

Cell culture methods can be useful in determining the biocompatibility of various components. The *in vitro* cytotoxic test [52] is an easy, repeatable, reproducible, appropriate and suitable *in vitro* assay for evaluating fundamental biologic characteristics. The colorimetric based MTT test can be used to evaluate cytotoxicity. The percentage of live cells and metabolic activity are determined by this test [53]. Following three days of incubation, the cytotoxicity of each antioxidant was evaluated by comparing the treated cells' viability with that of the control, which had been brought to 100% viability. The MTT assay's cytotoxicity findings on the viability of four different NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cell types with different antioxidants such as EDTA, Se, AA, and Zn are summarized in **Figure 1(A)-(D)**.

The viability of NIH-3T3 and SP/0Ag14 cells was found to be more effectively maintained by EDTA up to 40 μM, indicating that these cell types are not sensitive to the EDTA concentration being studied. Additionally, L929 cells have been shown to be insensitive to EDTA up to 10 μM, suggesting that EDTA concentrations of 20 μM or greater are cytotoxic to these cells. In contrast, EDTA has no effect on TF-1 cells up to 20 μM, indicating that TF-1 cells are cytotoxic at 40 μM or higher. The cells that exhibited the lowest cell viability in Se after three days of incubation were NIH-3T3, L929, TF-1, and SP/0Ag14. However, they remained non-toxic at concentration as high as 10 μM. It has been shown that Se concentrations of 10 μM or more drastically decrease NIH-3T3 cell viability, and that cytotoxicity increases with increasing Se concentrations.

Although TF-1 cells exhibit resistance to Se up to 20 μM, their viability was significantly reduced at 40 μM. On the other hand, SP/0Ag14 cells demonstrate sensitivity to Se at or above 20 μM and raising the concentration of Se to 20 μM and above also results in a reduction in cell viability. Up to 20 μM of selenium, the L929 cells were unaffected; however, at 40 μM, cell viability slightly decreased in comparison to the control. Se is a vital trace element that can modulate growth when added to cell culture medium [54]. However, depending on the concentration, it may be toxic to cells [34]. Given that Se acted as an antioxidant at lower

concentrations and as a pro-oxidant in greater amounts, our results suggest that Se acts as a pro-oxidant at greater concentrations in DMEM and RPMI media [55].

The cytotoxicity test of AA with NIH-3T3 cells viability showed that cells reduced their viability at or above 10 μM of AA. TF-1 cells are resistant to AA up to 20 μM, but at higher concentrations like 40 μM, the cell viability drastically dropped. Similarly, L929 and SP2/0-Ag14 cells were not susceptible to 40 μM of AA. However, past studies showed that AA at 10 nM - 1 mM caused human cells to undergo apoptosis [56] and that it was a significant regulator of mouse myeloma cell proliferation in an *in vitro* experiment [57]. These findings imply that the toxicity of AA largely depends on the kind of cell and its concentration, which means that high AA concentrations may be harmful for some cell types and harmless for others. Zinc cytotoxicity data indicate that NIH-3T3, TF-1, and Sp2/0-Ag14 cells are susceptible to zinc at 3 days, as demonstrated by their reduced viability at or above 5 μM. The L929 cell's viability was unaffected by the highest dose of zinc tested, 40 μM, in comparison to the corresponding control. This implies that Zn toxicity differs depending on the kind of cell [37], time of exposure, and concentration. However, many conflicting results about the toxicity of antioxidants have been published in the literature, necessitating further investigation. Therefore, it would make sense that variations in the results could be caused by the cell culture media and various techniques used during the experiment. Additionally, some investigators observed that the cytotoxicity assay results varied depending on the cell culture medium and the procedures used in the cytotoxic investigations [58].

According to the results of the cytotoxicity assay, after 3 days of exposure, the concentrations of antioxidants began to become cytotoxic at 5 μM and higher. To reduce cytotoxicity, 2.5 μM of noncytotoxic EDTA, Se, AA, and Zn concentration were used in proliferation assays. Thus, for three days, varying amounts of EGF (as detailed in the experimental section) with 2.5 μM antioxidants were used to investigate the proliferation of NIH-3T3, L929, TF-1, and Sp2/0-Ag14 cells independently. After a 3-day exposure to varying amounts of EGF with an extracellular concentration of 2.5 μM EDTA, Se, AA, and Zn, NIH-3T3, TF-1, and Sp2/0-Ag14 cells outperformed the corresponding positive control (**Figure 2(A)–(D)**). These antioxidants at 2.5 μM can prevent cellular damage by scavenging the medium's free radicals. Consequently, oxidative stress is likely to have decreased, and EGF has become more stable, retaining its reduced form and being available for cellular use. Although, after being treated for 3 days, L929 cells with varying amounts of EGF and an extracellular concentration of 2.5 μM of Se were observed to exhibit a slight cytotoxicity when compared to the positive control. Indicating that Se is most likely causing oxidative stress to L929 cells in MEM medium by acting as a pro-oxidant. However, the viability of L929 cells with other three antioxidants, such as EDTA, AA, and Zn, was slightly higher at higher EGF concentrations than with a positive control. This suggests that, while supplementing L929 cell culture with EGF and antioxidants can inhibit apoptosis, it cannot promote cell growth

in *in vitro* treatment.

Because the EGF samples were prepared in DMEM and RPMI medium, they needed to be free of any impurities that could interfere with EGF detection during the 25-minute run time. EGF was separated from DMEM and RPMI medium using the RP-HPLC method, with a single peak appearing at 9.2 minutes (**Figure 3(B)** and **Figure 3(D)**). It appears from these results that media components did not affect the EGF analysis under our experimental conditions (**Figure 3(A)** and **Figure 3(C)**). When compared to the control, EGF amounts were higher in samples of stability with EDTA, Se, AA, and Zn in DMEM (**Table 1**) and RPMI (**Table 2**) medium. Moreover, around 95% of EGF was recovered from both DMEM and RPMI medium following three days of incubation at 2 to 8˚C without the addition of antioxidants. In contrast, after being incubated at 37˚C without antioxidants, 75% of the EGF from the DMEM medium and 81% from the RPMI medium were recovered. This probably was happening because of the EGF protein becoming fragile at temperatures more than 2 to 8˚C, showing that it is a temperature-sensitive protein. In addition, EGF breakdown products like monomers, covalent dimerization, and high-molecular-weight molecules were not detected by RP-HPLC during the stability investigation. It is expected that more non-covalent aggregation will occur when EGF dissolution, complexation of ions, as well salting out, and neutrality of charge are restricted to the pH of the isoelectric. Therefore, it will be difficult to identify non-covalent aggregates due to the low pH of the organic solvent-containing mobile phase. However, there were no signs of oxidized or broken-down EGF in either the DMEM or the RPMI medium when the antioxidants EDTA, Se, AA, and Zn were present. These data show that an extracellular concentration of 2.5 μM of EDTA, AA, or Zn may stabilize EGF for up to three days in *in vitro* DMEM and RPMI medium conditions.

Furthermore, when 2.5 UM EDTA, Se, AA, and Zn were present in DMEM and RPMI medium, the peroxide level was determined. *In vitro* cell culture does not reduce the production of free radicals because the media used for cell culture lacks antioxidants, where cells are exposed to various physiochemical conditions [59], dissolved organic matter [60] is photochemically reduced, DMEM and RPMI medium contain metal ions [61], and oxygen (O_2) concentrations are higher than *in vivo* [15]. Thus, proteins can be oxidized by aqueous solutions due to radical species [62]. Therefore, the purpose of this study was to use the modified IDF peroxide method with an extracellular concentration of 2.5 μM of four different antioxidants, namely EDTA, Se, AA, and Zn, to evaluate the peroxide levels in DMEM and RPMI medium with 50 μg/mL EGF separately. The findings have been compared with a control group that received no antioxidants. The results show that each antioxidant reduced peroxide level in both DMEM **(Figure 5(A)** and **Table 3)** and RPMI (**Figure 5(B)** and **Table 4)** medium**.** The results of the study show that an independent 2.5 μM of the antioxidants EDTA, Se, AA, and Zn may decrease the level of peroxide by removing free radicals and chelated ions of metal found in DMEM and RPMI medium. More *in vitro* investigation is required to fully understand how the anti-

oxidants reduce peroxide level in DMEM and RPMI medium. According to the study's findings, the antioxidants employed in the present study can lower the peroxide levels in cell culture media like DMEM and RPMI.

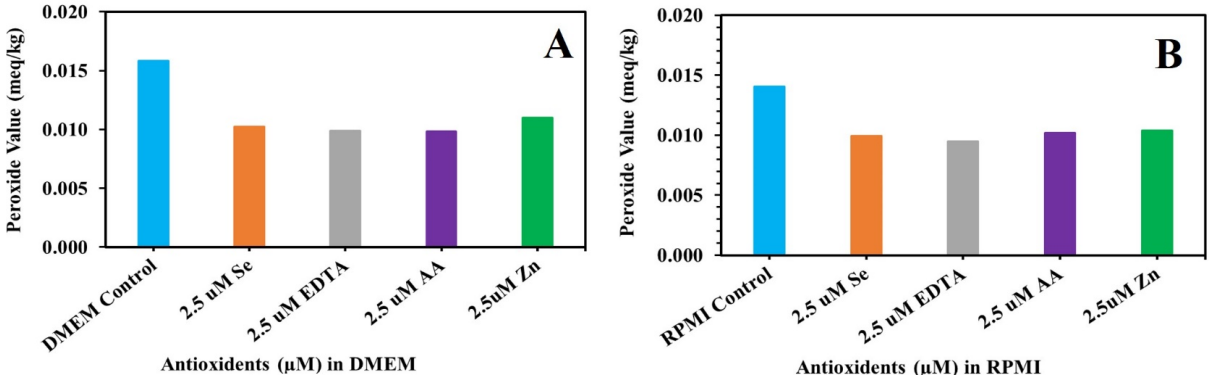

Figure 5. Antioxidants such EDTA, Se, AA, and Zn were used to measure the level of peroxide in DMEM and RPMI serum-free medium. Separately a 50 ng/mL EGF solution was prepared in DMEM and RPMI medium with 2.5 µM of EDTA, Se, AA, and Zn. After that, the solution was incubated at 37˚C for three days. The peroxide content was measured in milliequivalents per kilogram. In DMEM (A) and RPMI (B) medium, the antioxidants EDTA, Se, AA, and Zn reduced peroxide levels compared to the control. The result represents the average of three separate experiments.

5. Conclusion

When added to the DMEM and RPMI medium individually, antioxidants at 2.5 µM extracellular concentrations were found to improve cell proliferation, stabilize EGF, and lower peroxide levels. This method may also be used in pharmaceutical formulations, parenteral formulations, and other solution-based EGF solutions. To completely understand the function of antioxidants in cell culture medium, more investigation is required.

Authors Contributions

A. S. Prakasha Gowda conceptualized, designed, executed the experiments, carried out data analysis and wrote the manuscript. The manuscript was reviewed by Andrew D. Schaefer and Terry K. Schuck. All authors granted their approval for publication.

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Conflicts of Interest

The authors confirm that this article content has no conflict of interest. This arti-

cle does not contain any studies with human or animal subjects. The company had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results, in both Disclosure form and manuscript file.

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