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Instability of Fibroblast Growth Factor 2 in Cell Culture Media

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ABSTRACT

Fibroblast growth factor 2 (FGF-2) is a key signaling molecule involved in wound healing, tissue remodeling, and the regulation of cell proliferation. Despite its important biological roles, the use of FGF-2 in cell culture and therapeutic settings is limited by its poor stability in aqueous environments. Three different antioxidants were selected and screened in this study for their ability to enhance the stability of FGF-2 in cell culture applications. BALB/3T3 and NIH/3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented individually with disodium ethylenediaminetetraacetic acid dihydrate (EDTA), sodium selenite (Se), or zinc chloride (Zn), each at a final concentration of 0.1 μM. Cytotoxicity and cell proliferation were subsequently assessed using the MTT assay. The cells were then cultured at 37˚C for three days to assess the biological effects of antioxidant supplementation. All three antioxidants individually enhanced the proliferation of both cell lines without inducing cytotoxicity. These findings suggest that antioxidants may represent a promising approach for improving *vitro* cell growth. Therefore, antioxidants were evaluated in parallel with FGF-2 by adding them separately to DMEM and Roswell Park Memorial Institute (RPMI) 1640 serum and cells free media under identical conditions to determine whether their proliferative effects were associated with improved FGF-2 stability. The stability of FGF-2 in each medium was assessed using reversed phase high performance liquid chromatography (RP-HPLC). The results show that, even in the presence of antioxidants, FGF-2 remained unstable at 37˚C in both DMEM and RPMI media, indicating that these antioxidants do not confer stability to FGF-2 in either medium.

1. INTRODUCTION

The most studied member of the FGF family is FGF-2, also known as basic fibroblast growth factor (bFGF). It is a single chain, non-glycosylated polypeptide. In both healthy and pathological conditions, FGF-

2 plays an essential role in several biological functions, including regulating cell division and proliferation [1]. These characteristics have made FGF-2 a desirable option for pharmaceutical uses such as cancer treatment, tissue repair, and regenerative medicine [2]. Several physiological processes, such as mitogenesis, cell migration, morphogenesis, angiogenesis, organ development, tissue regeneration, and wound healing, are regulated by FGF-2 [3]. Elevated levels of FGF-2 have been detected in acute wounds and during tissue repair [4], as well as in burns [5] and limb ischemia [6]. In *in vitro*, FGF-2 acts as a growth factor [7], promoting proliferative effects in NIH/3T3 and BALB/3T3 cells [8, 9].

The intrinsic instability of FGF-2 in aqueous solutions has made it challenging to formulate into effective therapeutic products because it degrades quickly [10]. It has been demonstrated that heparin supplementation stabilizes FGF-2, protecting it from acid, proteases, heat, and trypsin [11, 12]. However, the potent anticoagulant activity of heparin restricts its application as an antioxidant and raises significant safety concerns [11, 12]. The instability of FGF-2 solutions impacts cell proliferation, therapeutic effectiveness, and purity; thereby, ensuring its *in vitro* stability is essential for the successful development of medicinal formulations and cell culture applications.

Furthermore, solution conditions that maintain native conformational integrity impact protein stability and functionality. Rapid changes in physiological conditions typically cause degradation, denaturation, or aggregation, resulting in the permanent loss of biological activity. In addition, reactive oxygen species (ROS) dramatically affect protein stability by oxidizing proteins and impairing cellular integrity [13]. Antioxidants enhance the stability and solubility of proteins in liquid formulations, thereby preserving their biological activity and structural integrity. In pharmaceutical preparations, antioxidants are classified as inactive components and are routinely incorporated into protein formulations to improve stability and extend shelf life. The selection of antioxidants is guided by the intended therapeutic application and the specific experimental conditions [14, 15].

The stability of FGF-2 in aqueous solutions has been widely studied [16, 17], with a focus on the effect of antioxidants minimizing chemical changes. Pharmaceutically authorized antioxidants, such as salts, polymers, proteins, and amino acids [18], help to stabilize proteins usually through ionic interactions. Similarly, carbohydrates improve stability by raising surface tension and solution viscosity, which reduces protein aggregation [18]. Furthermore, the addition of human serum albumin (HSA), or bovine serum albumin (BSA) improves stability through hydrophobic, ionic, and electrostatic interactions [18, 19]. Most studies have highlighted FGF-2 stability as an important factor affecting pharmaceutical formulations' viability. As of currently, there is no established method for stabilizing FGF-2 in aqueous solutions, and there is no evidence of antioxidant selection that is especially suited to FGF-2. Little is known about the stability of FGF-2 in cell culture medium, especially at physiological temperature of 37˚C. The stability of FGF-2 in widely used culture media such as DMEM and RPMI remains uncertain. Therefore, understanding the stability of FGF-2 in cell culture media particularly in the presence of antioxidants is essential, as this simple and cost-effective strategy has the potential to influence both FGF-2 stability and cellular responses.

In the present study, three distinct antioxidants such as EDTA [20], Se [21], and Zn [22] were selected and investigated for their effects on the stability of FGF-2 in both Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium. These antioxidants were selected because of their proven antioxidant and radical-scavenging characteristics and are expected to improve cell proliferation and decrease oxidative damage to FGF-2 in culture medium. The resulting information is important in optimizing FGF-2 formulations for *in vitro* applications. In this study, the effects of three antioxidants EDTA, Se, and Zn on FGF-2 stability were independently evaluated in serum and cell free DMEM and RPMI media using RP-HPLC [23, 24]. Additionally, the biological activity of FGF-2, including cytotoxicity and cell proliferation responses, was assessed *in vitro* by the MTT assay using BALB/3T3 and NIH/3T3 fibroblast cell lines to determine how these antioxidants influence FGF-2 function.

2. MATERIALS AND METHODS

2.1. Materials

Lyophilized FGF-2 was reconstituted at 1 mg/mL in 1 mL of Milli-Q water for stability testing and

experimental use. The stock solution was diluted in Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) medium to a working concentration of 50 μg/mL. Unused aliquots of the FGF-2 stock were stored at −20˚C. Thermo Fisher Scientific (Cranbury, NJ, USA) supplied the Fetal Bovine Serum (FBS) and 0.25% trypsin-EDTA solution. Disodium ethylenediaminetetraacetic acid dihydrate, sodium selenite, zinc chloride, Cell Proliferation Kit I (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and phosphate buffer saline (PBS) Buffer 1X, pH 7.4 were obtained from Millipore Sigma (Burlington, MA, USA). Individual stock solutions of these compounds were prepared in Milli-Q water and used as antioxidant sources in assays, cell proliferation studies, FGF-2 stability testing and *in vitro* cytotoxicity assays. Acetonitrile was purchased from Fisher Scientific (Cranbury, NJ, USA), Dimethyl sulfoxide (DMSO), orthophosphoric acid (HPLC grade) from Sigma Aldrich (Burlington, MA, USA), and 10N hydrochloric acid from Ricca Chemical Company (Houston, TX, USA). Waters Corporation (USA) provided LectraBond cap preslit PTFE/silicone septa, and Ricca supplied 12 × 32 mm glass screw-neck vials with quick-thread closures. Mouse fibroblast cell lines BALB/3T3 and NIH/3T3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI 1640 medium and DMEM were purchased from Life Technologies Corporation (Grand Island, NY, USA). Milli-Q water was prepared in-house using Millipore equipment (Millipore, Milford, MA, USA). Cell culture flasks were obtained from Corning (Corning, NY, USA). Stock solutions and reagents were freshly prepared in DMEM and RPMI prior to use.

2.2. Methods

2.2.1. Cell Type and Cell Culture Conditions

Mouse fibroblast cell lines BALB/3T3 and NIH/3T3 were cultured in DMEM supplemented with 10% FBS and 0.01 mg/mL streptomycin sulfate, 100 U/mL penicillin (Complete medium), and Cultures were maintained in a humidified incubator at 37˚C with 5% CO_2, and the medium was replaced every three days. Cell viability was monitored using the dye exclusion method [25], and cultures consistently maintained about 90% viability throughout the study.

Because both BALB/3T3 and NIH/3T3 are adherent cell lines, sub-culturing was performed prior to experimental setup. After removal of the spent medium, cells were washed with 10 mL of phosphate-buffered saline (PBS, pH 7.4) and subsequently treated with 4 mL of 0.25% trypsin-EDTA prepared in Hanks' balanced salt solution lacking calcium and magnesium. Cells were allowed to detach for 3 - 4 minutes at room temperature, after which the cell suspension was collected, resuspended in complete medium, and counted. Cells were then seeded at a density of 4000 cells per well into 96-well plates, with each condition plated in duplicate wells. After overnight adhesion, the medium was replaced with fresh complete medium before the addition of antioxidants and FGF-2 for cytotoxicity and proliferation assays.

2.2.2. Cell Cytotoxicity in the Presence of Antioxidants

The cytotoxicity of the antioxidants EDTA, Se, and Zn toward BALB/3T3 and NIH/3T3 mouse fibroblast cell lines was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay measures cell viability based on the capacity of mitochondrial succinate dehydrogenase in metabolically active cells to reduce the yellow tetrazolium substrate to an insoluble purple formazan product. The resulting formazan was quantified spectrophotometrically at 570 nm, with absorbance values directly proportional to the number of viable cells.

For cytotoxicity assessment, BALB/3T3 and NIH/3T3 cells were seeded into 96-well plates at 4000 cells per well in 50 μL of cell suspension in complete medium. Following overnight attachment, 50 μL of complete medium containing three independent concentration series of each antioxidant were added separately. The concentrations tested were 0.04, 0.08, 0.156, 0.313, 0.625, 1.25, 2.5, and 5.0 μM; 0.008, 0.016, 0.032, 0.063, 0.125, 0.25, 0.5, and 1.0 μM; and 0.001, 0.002, 0.005, 0.01, 0.04, 0.1, 0.2, and 0.4 μM. Control cell wells received complete medium without antioxidants while wells containing medium alone served as reagent blanks. Plates were incubated for three days at 37˚C in a humidified atmosphere of 5% CO_2.

After exposure, 10 μL of MTT solution (5 mg/mL in PBS, pH 7.4) was added to each well, followed by

a 3-hour incubation under the same conditions. The medium was then carefully aspirated without disturbing the formazan crystals, and 200 µL of DMSO was added to solubilize the crystals. The plates were then incubated for 30 minutes at 37˚C to ensure complete dissolution. Absorbance was measured at 570 nm with background correction using blank wells containing only MTT reagent and DMSO. Cytotoxicity was reported as percent cell viability, calculated using optical density measurements from the test, control, and blank wells according to the previously described method [26]. The data represents the mean viability percentage from three independent experiments.

2.2.3. Investigating How Antioxidants Influence FGF-2 Mediated Cell Proliferation

FGF-2 induced proliferation in the presence of selected antioxidants was evaluated using the MTT assay in BALB/3T3 and NIH/3T3 fibroblast cell lines. Cells were seeded into 96 well plates at a density of 4000 cells per well in 50 µL of complete medium and incubated overnight to allow the cells attachment.

On the following day, 50 µL of complete medium containing three independent series of increasing FGF-2 concentrations ranging from 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 12.5, and 25.0 ng/mL; 0.005, 0.01, 0.02, 0.05, 0.1, 0.5, 1.0, and 2.5 ng/mL; and 0.004, 0.008, 0.016, 0.032, 0.0625, 0.125, 0.25, and 0.5 ng/mL each supplemented with 0.1 µM of each individual antioxidant (EDTA, Se, and Zn), was added to the wells. Control wells contained cells in complete medium without FGF-2 or antioxidants, and wells containing 100 µL of complete medium served as reagent blanks.

Plates were incubated for 3 days at 37˚C in a humidified atmosphere with 5% CO_2. After treatment, 10 µL of MTT solution (5 mg/mL in PBS, pH 7.4) was added to each well, including controls and blanks, followed by a 3 h incubation under the same conditions. The medium was then carefully aspirated to avoid disturbing the formazan crystals, and 200 µL of DMSO was added to each well to solubilize the crystals. Plates were incubated for an additional 30 min at 37˚C to ensure complete dissolution. Absorbance was measured at 570 nm with background subtraction using blank wells.

Cell proliferation was expressed as percent viability based on optical density values from treated, control, and blank wells, calculated as described previously [26]. Data represents viability values from three independent experiments.

2.2.4. Reverse Phase HPLC Conditions

RP-HPLC analysis was performed [26] using a system equipped with a quaternary pump, online degasser, thermostatted column compartment, autosampler with injection loop, and variable-wavelength UV detector. Sample vials were maintained at 5˚C in the autosampler, and all chromatographic analyses were conducted at room temperature. Separation was achieved using a Zorbax 300SB-CN column (4.6 × 150 mm, 3.5 µm), maintained at 20˚C.

The mobile phase consisted of solvent A (0.1% orthophosphoric acid in water) and solvent B (0.1% orthophosphoric acid in acetonitrile). FGF-2 was eluted using a linear gradient from 5% to 40% solvent B. The flow rate was set to 0.6 mL min, the injection volume was 5 µL, and detection was carried out at 214 nm. Chromatographic data, including peak integration, peak area quantification, and retention time, were processed using Empower software.

2.2.5. Analyzed Stability of FGF-2 in DMEM and RPMI Medium with Antioxidants

To examine the impact of the antioxidants EDTA, Se, and Zn on FGF-2 stability, FGF-2 stock solutions were prepared at a concentration of 200 µg/mL in serum and cell free DMEM and RPMI media. Each antioxidant was added individually to the stock solutions at a final concentration of 0.1 µM. For each medium, two corresponding control vials containing FGF-2 at 200 µg/mL without antioxidants were prepared. One test vial and one control vial for each medium were incubated at 37˚C for defined time intervals (1, 2, 6, 12, 24, 48, and 72 h), while the second control vial from each medium was stored at 2˚C - 8˚C for the same time points. Three independent vials were prepared for each condition at the specified time points. At each time FGF-2 stability was assessed relative to freshly prepared FGF-2 from its stock solution, and the percentage of FGF-2 recovered in DMEM and RPMI was quantified using Empower software.

In Empower, Percent Recovery is calculated using a custom field with the formula:

(Amount/Theoretical Amount) × 100

Amount: The measured or calculated amount from the sample result. **Theoretical Amount**: The expected or nominal amount (based on standard concentration). The result is expressed as a percentage, representing how much of the expected amount was recovered.

2.2.6. Statistical Data Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare differences between groups. When significant differences were detected, post hoc comparisons were conducted using Tukey's test. In all analyses, p-values < 0.05 were considered statistically significant, and p-values < 0.01 were considered highly significant. Data are presented as mean ± standard error (SE). Exact p-values are reported in the respective figure legends.

3. RESULTS

3.1. Cells Cytotoxicity Depends on Antioxidants Concentration

The cytotoxic effects of the antioxidants EDTA, Se, and Zn were evaluated in BALB/3T3 and NIH/3T3 fibroblast cell lines by exposing the cells separately to increasing concentrations of each antioxidant (0.04, 0.08, 0.16, 0.31, 0.62, 1.25, 2.5, and 5.0 μM). Complete medium alone was used for the blank, and cells cultured in complete medium without any antioxidants served as the controls. Cytotoxicity was assessed for 3 days using the MTT colorimetric assay. The resulting cell viability profiles for BALB/3T3 and NIH/3T3 cells are presented in (**Figures 1(A)-(F)**) respectively.

After 3 days of exposure, both fibroblast cell lines exhibited increased cell growth relative to their untreated controls at the lower antioxidant concentrations. However, a clear concentration dependent decline in cell growth was observed across the tested range of 0.031 - 5.0 μM. These results indicate that BALB/3T3 and NIH/3T3 cells display partial resistance to moderate antioxidant concentrations, although viability progressively decreased as concentrations increased. Notably, both cell lines showed pronounced sensitivity to the highest Zn concentration tested (**Figures 1(A)**) and (**Figure 1(B)**). Following antioxidant treatment, light microscopy observation showed noticeable morphological changes in both cell lines, with fibroblasts taking on a rounded appearance instead of their characteristic spindle-shaped morphology (data not shown). These changes are consistent with cytotoxic responses and indicate that both fibroblast cell lines are susceptible to high antioxidant concentrations [27].

To further investigate the effects of lower antioxidant concentrations, EDTA, Se, and Zn were independently evaluated at concentrations of 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, and 1.0 μM. At these lower concentrations, both BALB/3T3 (**Figure 1(C)**) and NIH/3T3 (**Figure 1(D)**) cells show enhanced growth. However, morphological abnormalities persisted at the upper end of 1.0 μM from each antioxidant, where a substantial proportion of cells appeared rounded (data not shown). These observations suggest that even low antioxidant concentrations can elicit detectable morphological alterations in fibroblasts.

An additional range of lower concentrations (0.001, 0.002, 0.005, 0.01, 0.04, 0.1, 0.2, and 0.4 μM) was investigated to further determine the ideal range of antioxidants. In these conditions, both fibroblast cell lines grew more. Furthermore, at these lowest concentrations, both BALB/3T3 (**Figure 1**)) and NIH/3T3 (**Figure 1(F)**) cells maintained high viability and their characteristic spindle-shaped morphology (data not shown).

Since both fibroblast cell lines demonstrated sensitivity to EDTA at higher concentrations, BALB/3T3 and NIH 3T3 cells are likely more susceptible to EDTA-induced cytotoxicity at higher concentrations. This result shows that different cell types and concentrations of EDTA can have different effects on cells [26], [28]. In the same way, it is known that Se's biological activity depends on concentration [29]. Higher concentrations of Se can cause oxidative stress and increase the production of ROS, whereas lower concentrations have been demonstrated to promote cell proliferation [30]. Similarly, a concentration dependent

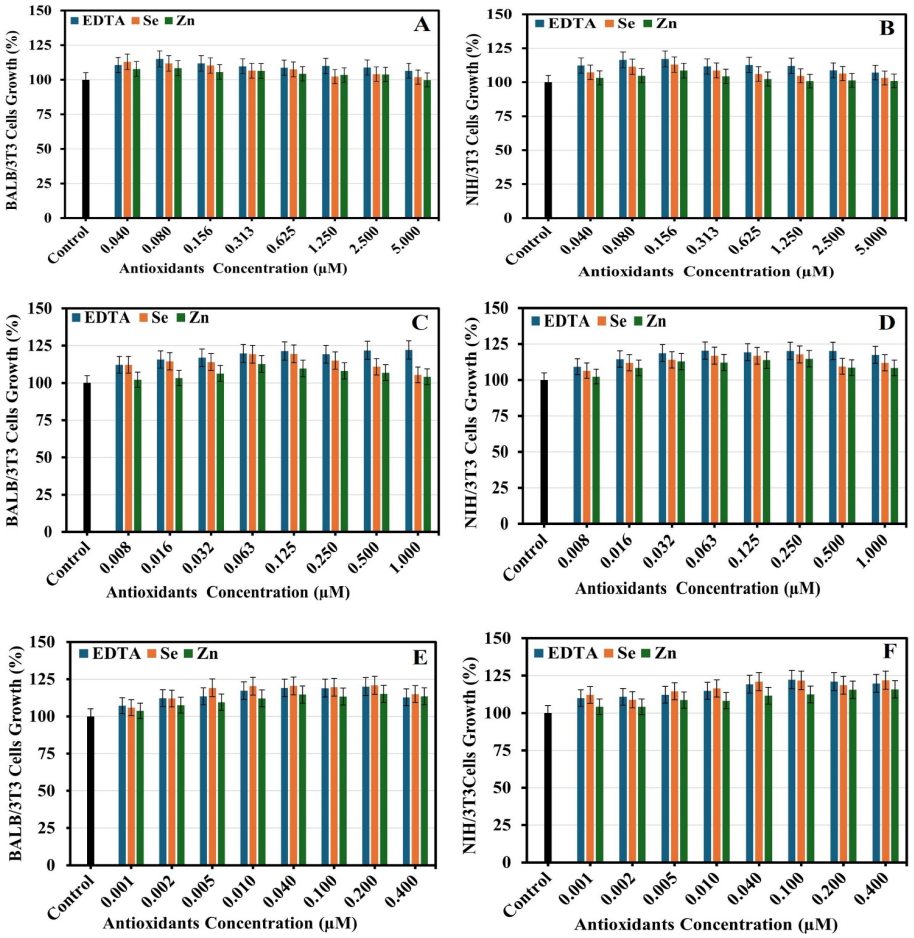

Figure 1. The cytotoxic effects of EDTA, Se, and Zn on BALB/3T3 and NIH/3T3 cells. BALB/3T3 and NIH/3T3 cells were exposed to increasing concentrations of EDTA, Se, or Zn (0.04 - 5 μM) for 3 days at 37˚C. Cell viability was determined using the MTT assay and the result was compared to untreated controls. Error bars indicate the mean ± SE derived from three independent experiments, each conducted in duplicate wells. Both BALB/3T3 (A) and NIH/3T3 (B) cells exhibited concentration-dependent reductions in viability, demonstrating increased sensitivity to the antioxidants at higher concentrations. At these elevated levels, cells appeared rounded and showed marked alterations in morphology. To further evaluate the effects of lower antioxidant concentrations, cells were treated with EDTA, Se, or Zn in the range of 0.008 - 1.0 μM. At these concentrations, both BALB/3T3 (C) and NIH/3T3 (D) cells displayed enhanced growth but remained sensitive to the upper end of the antioxidant's concentration. Morphological abnormalities persisted, with a notable proportion of rounded cells, indicating that even low antioxidant concentrations induced detectable changes in fibroblast morphology. Finally, optimized lower concentrations of EDTA, Se, and Zn (0.001 - 0.4 μM) were tested in BALB/3T3 (E) and NIH/3T3 (F) cells. At these concentrations, both cell lines showed concentration dependent increases in growth, and cell morphology appeared normal by light microscopy observation. Controls cells were maintained separately. Statistical analysis was performed using one-way analysis of variance (ANOVA). Cytotoxicity values for antioxidant treated groups were considered significantly different from those obtained with 0 μM antioxidant (control, defined as 100% cell viability in control medium) at $p < 0.05$.

reduction in cell viability was found in cytotoxicity tests of BALB/3T3 and NIH/3T3 cells exposed to higher Zn concentrations. Because viability gradually declined with increasing Zn concentration after three days of Zn treatment, it appears that Zn cytotoxicity varies by cell type [31] and has a concentration dependent response [32]. In the present study, high EDTA [33], Se [30], and Zn [34] concentrations can induce cytotoxicity by causing oxidative stress in both tested BALB/3T3 and NIH/3T3 cells, resulting in reduced viability and noticeable altered morphology when compared to their corresponding controls. In conclusion, it was observed that both fibroblast cell lines were susceptible to higher concentrations of all three of the tested antioxidants.

According to our earlier study [26], an extracellular concentration of 2.5 μM EDTA, Se, and Zn were used in cell proliferation assays. In this study, microscopic analysis showed that BALB/3T3 or NIH/3T3 cell viability and morphology were unaffected by exposure to 0.1 μM of EDTA, Se, or Zn separately. Both cell lines maintained their distinctive spindle-shaped morphology at this concentration and showed higher viability levels than untreated controls. Based on these findings, 0.1 μM was identified as a non-cytotoxic concentration for each antioxidant and was consequently selected as the working concentration for all subsequent cell proliferation and FGF-2 stability experiments.

3.2. Antioxidants in Combination with FGF-2, Induced Cell Proliferation

Both NIH/3T3 and BALB/3T3 fibroblast cell lines require FGF-2 for optimal *in vitro* growth [8]. A range of FGF-2 concentrations were assessed in this study with the objective to determine which concentration most effectively promotes cell proliferation. BALB/3T3 and NIH/3T3 cells were seeded separately into 96-well plates at a density of 4,000 cells per well in 50 μL of complete medium. After overnight incubation to allow for cell attachment, 50 μL of FGF-2 solutions containing 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 12.5, or 25 ng/mL were added. Control wells for each cell line contained cells cultured in complete medium without FGF-2, while wells containing 100 μL of complete medium as blanks.

The plates were then incubated for 3 days at 37˚C with 5% CO_2 in a humidified incubator. The proliferation responses of BALB/3T3 and NIH/3T3 cells to the tested FGF-2 concentrations are shown in (**Figure 2(A)**). BALB/3T3 cells exhibited increased proliferation relative to their respective control; however, proliferation decreased progressively as the FGF-2 concentration increased. NIH/3T3 cells also demonstrated a modest increase in growth compared with control cells, but overall proliferation remained consistently lower than that observed for BALB/3T3 cells and showed minimal variability across the FGF-2 concentration range. These results (**Figure 2(A)**) suggest that NIH/3T3 cells may exhibit greater sensitivity to elevated FGF-2 levels.

FGF-2 has been investigated over two more concentration ranges to further optimize the concentration: 1) 0.005, 0.01, 0.02, 0.05, 0.1, 0.5, 1.0, and 2.5 ng/mL, and 2) 0.004, 0.008, 0.016, 0.023, 0.063, 0.125, 0.25, and 0.5 ng/mL. BALB/3T3 and NIH/3T3 cells were separately seeded into 96-well plates at 4,000 cells per well in 50 μL of complete media for both FGF-2 concentration ranges. After that, the cells were kept in a humidified incubator for three days at 37˚C with 5% CO_2. The results of the proliferation are shown in (**Figure 2(B)**) and (**Figure 2(C)**). As shown in (**Figure 2(B)**), BALB/3T3 and NIH/3T3 cells both multiplied more than their corresponding controls when the FGF-2 concentration was reduced. However, at concentrations above 0.5 ng/mL, proliferation of both cell lines began to decrease, suggesting that higher FGF-2 levels may activate inhibitory signaling pathways in both cell types.

In agreement with these observations, the data shown in (**Figure 2(C)**) indicate that further reductions in FGF-2 concentration during the three-day incubation period increased the proliferation of both BALB/3T3 and NIH/3T3 cells in a concentration dependent manner. A slight decrease in proliferation was observed at 0.5 ng/mL FGF-2; however, both cell lines exhibited their highest proliferative response at 0.125 ng/mL, at which cell viability remained high. Although BALB/3T3 cells consistently demonstrated greater proliferation overall, NIH/3T3 cells showed lower proliferative responses across all FGF-2 concentrations tested. This pattern suggests that FGF-2 may not function as a potent mitogen for NIH/3T3 cells, or alternatively, that NIH/3T3 cells may require a different optimal concentration range to achieve maximal growth. Based on

these findings, the FGF-2 concentration range of 0.004 - 0.5 ng/mL was selected as the working range for both BALB/3T3 and NIH/3T3 cells in all subsequent proliferation assays involving antioxidant treatments.

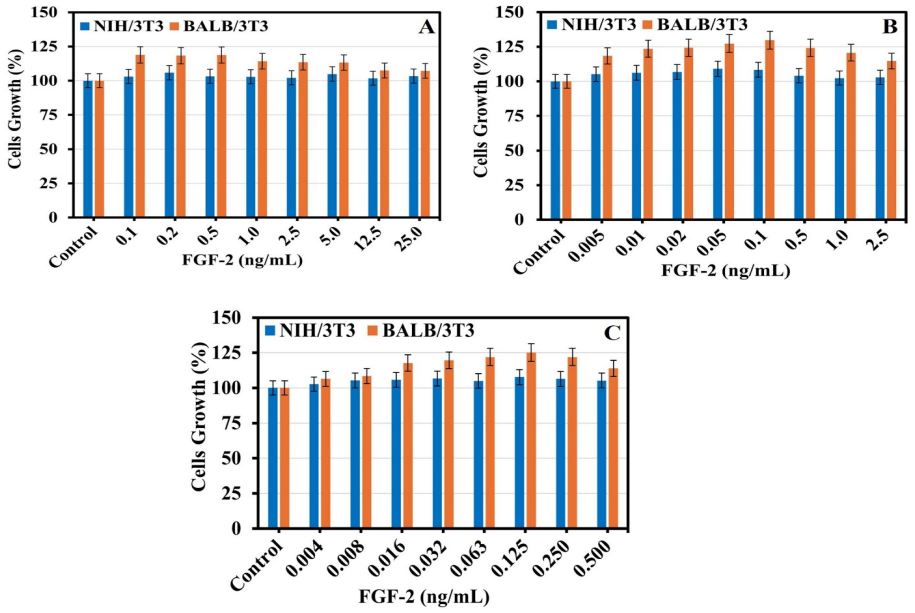

Figure 2. FGF-2 concentration optimization for BALB/3T3 and NIH/3T3 cells growth. BALB/3T3 and NIH/3T3 cells were treated with a range of FGF-2 concentrations to determine the optimal dose for proliferation. Error bars indicate the mean ± SE derived from three independent experiments, each conducted in duplicate wells. Initial testing with 0.1 - 25 ng/mL FGF-2 (A) showed increased proliferation at lower concentrations, followed by reduced cell growth and noticeable changes in cells morphology as the concentration increased. Therefore, cells were subsequently treated with an intermediate range of 0.005 - 2.5 ng/mL FGF-2 (B). In this range, both cell lines exhibited enhanced proliferation, although morphological changes, including rounding, were still observed. BALB/3T3 and NIH/3T3 cells were further treated with 0.004 - 0.5 ng/mL FGF-2 (C) to determine a concentration range that promoted proliferation while preserving normal cell morphology. At these lower concentrations, both cell lines showed increased proliferation with morphology appearing normal under light microscopy. All the results were compared with their corresponding controls. Statistical analysis was performed using one-way analysis of variance (ANOVA). Proliferation values for FGF-2 treated groups were considered significantly different from those obtained with 0 ng/mL FGF-2 (control, defined as 100% cell growth in control medium) at $p < 0.05$.

In later experiments, we investigated whether a specific antioxidant could enhance the stability of FGF-2 and thereby promote the proliferation of BALB/3T3 and NIH/3T3 cells. A series of FGF-2 concentrations (0.004, 0.008, 0.016, 0.023, 0.063, 0.125, 0.25, and 0.5 ng/mL) was prepared, and each concentration was supplemented individually with 0.1 μM EDTA, Se, and Zn before being applied to BALB/3T3 and NIH/3T3 cultures. The culture medium alone served as the blank, and separate controls for each cell line were maintained in complete medium lacking both FGF-2 and antioxidants. All cultures were incubated for three days at 37˚C in a humidified atmosphere containing 5% CO_2.

The proliferative responses of BALB/3T3 and NIH/3T3 cells to combined FGF-2 and antioxidant treatments are presented in (Figure 3(A)) and (Figure 3(B)). Supplementation with 0.1 μM EDTA, Se, and Zn

individually enhanced the proliferation of BALB/3T3 cells in an FGF-2 dependent manner compared with untreated controls (Figure 3(A)). However, under the same treatment conditions, NIH/3T3 cells showed a modest increase in proliferation (Figure 3(B)), suggesting that the antioxidant and FGF-2 combinations also stimulated their growth. All these results demonstrate that both cell lines need FGF-2 to promote *in vitro* proliferation and that, as compared to control cultures, antioxidant supplementation boosts their proliferative responses. The results imply that by reducing oxidative stress in the culture environment, these lowest antioxidant concentrations may stabilize FGF-2 and contribute to the observed increases in cell proliferation.

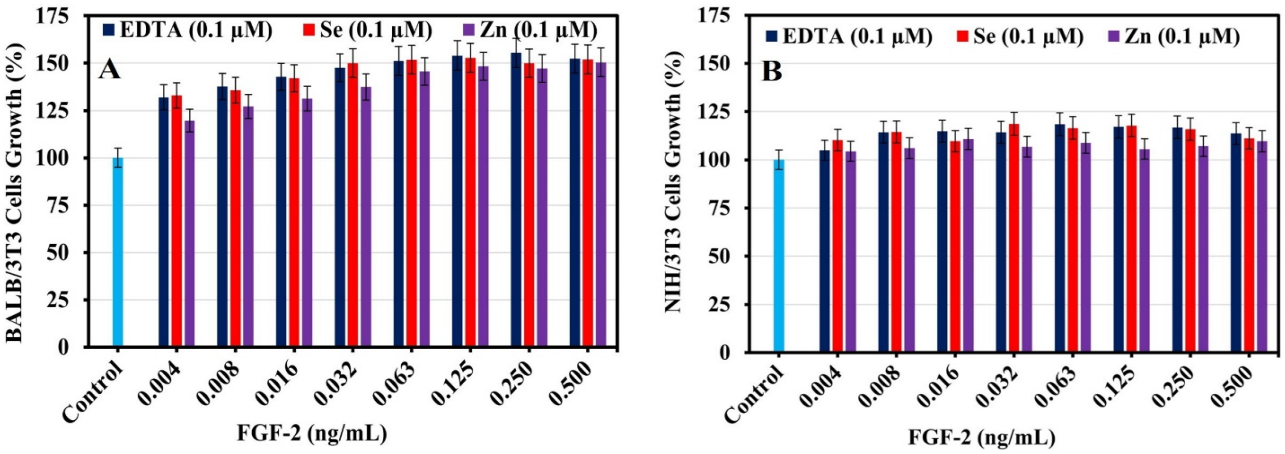

Figure 3. Antioxidants effects on BALB/3T3 and NIH/3T3 cell proliferation mediated by FGF-2. BALB/3T3 and NIH/3T3 cells were treated with increasing concentrations of FGF-2 (0.00 - 0.5 ng/mL) in the presence of individually supplemented antioxidants (0.1 μM EDTA, Se, or Zn). Error bars indicate the mean ± SE derived from three independent experiments, each conducted in duplicate wells. Both BALB/3T3 (A) and NIH/3T3 (B) cells exhibited enhanced proliferation with normal cell morphology under these conditions. Cell proliferation at each FGF-2 concentration, relative to the untreated control, was quantified for each antioxidant. Data represents mean percentage proliferation from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA). Proliferation values for antioxidants treated with FGF-2 groups were considered significantly different from those obtained with 0 ng/mL and 0 μM antioxidant (control, defined as 100% cell viability in control medium) at $p < 0.05$.

3.3. FGF-2 Is Unstable in DMEM and RPMI Medium Supplemented with Antioxidants

To determine if the observed increase in cell proliferation was related to enhanced FGF-2 stability in the presence of antioxidants such as EDTA, Se, and Zn, which have demonstrated radical-scavenging and antioxidative properties, each antioxidant was evaluated individually. The stability of FGF-2 in serum and cell free DMEM and RPMI media was supplemented with these antioxidants. Protein stability in aqueous solutions at 37˚C remains challenging to evaluate accurately due to the high variability and limited precision of currently available bioassays [18, 35]. Therefore, analytical RP-HPLC was used to assess stability, according to established growth factor measurement procedures [26].

The effects of column type, mobile-phase composition, and detector settings were systematically examined. Maintaining the column temperature at 20˚C provided sufficient resolution to separate all sample components within a 30-min run. Under these optimized conditions, FGF-2 produced a single peak at a retention time of 8.7 min with high sensitivity and reproducibility. Additionally, 5 μL aliquots of DMEM and RPMI were analyzed separately under the same RP-HPLC conditions to check for the interference from

media ingredients (data not shown). There were no peaks at the FGF2 retention time, indicating that there were no co-eluting contaminants in the FGF-2 signal and that the components of the culture media did not affect detection. Individually, 0.1 μM EDTA, Se, and Zn was added to a 200 μg/mL FGF-2 solution prepared in serum and cell free DMEM or RPMI. All FGF-2 stability samples were prepared and subsequently incubated at 37˚C for various time intervals. Following incubation, each sample was analyzed by RP-HPLC, and the percentage of recovered FGF-2 was compared with freshly prepared FGF-2 samples and corresponding controls. The analytical results demonstrated that supplementation with 0.1 μM EDTA, Se, and Zn did not enhance FGF-2 stability in either DMEM or RPMI. Detailed stability results for FGF-2 in the presence of each antioxidant are presented in Table 1 and (Figure 4(A)) for DMEM, and in Table 2 and (Figure 4(B)) for RPMI.

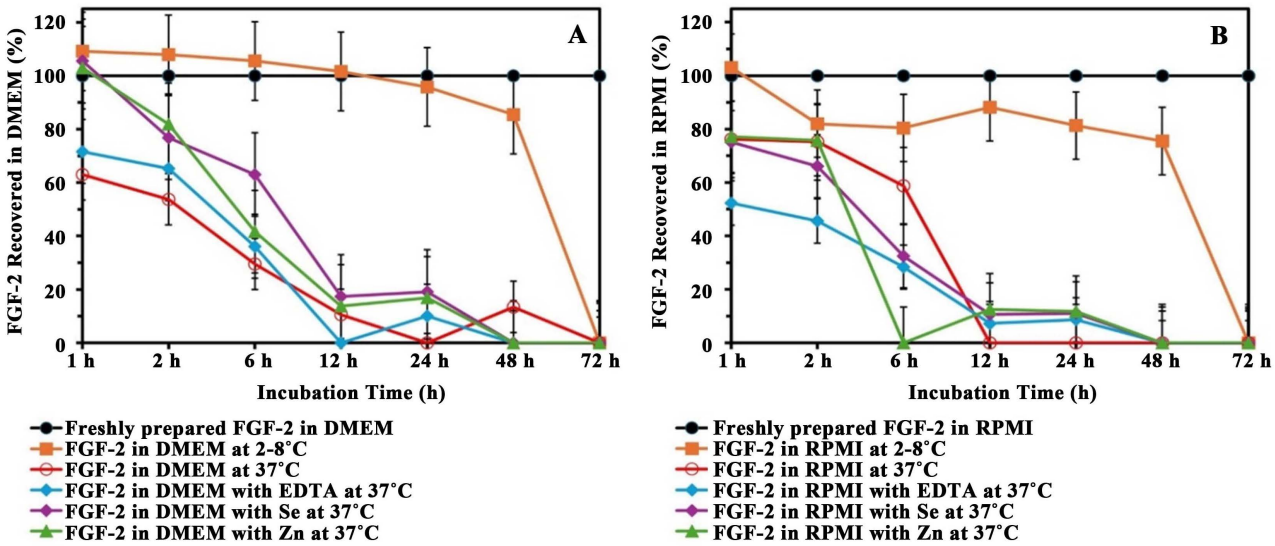

Figure 4. Recovery of FGF-2 in DMEM and RPMI after treatment with antioxidants, determined by RP-HPLC analysis. Error bars indicate the mean ± SE derived from three independent experiments. FGF-2 solutions (200 μg/mL) were prepared in DMEM (A) and RPMI (B) supplemented individually with 0.1 μM EDTA, Se, or Zn. For the control samples, 200 μg/mL FGF-2 was prepared in each medium without antioxidants. Two sets of control vials were included: one stored at 2˚C - 8˚C, the other control and all test samples were incubated at 37˚C for designated time points. Then each sample and control solutions was analyzed independently by RP-HPLC, and FGF-2 percent recovery was quantified using Empower software. Freshly prepared FGF-2 was defined as 100% recovery and served as the reference for calculating the recovery of all other samples. Statistical analysis was performed using one-way analysis of variance (ANOVA). FGF-2 stability values in DMEM and RPMI media for antioxidant-treated groups were considered significantly different from those obtained with 0 ng/mL FGF-2 and 0 μM antioxidant (control, defined as freshly prepared FGF-2 representing 100% recovery), at $p < 0.01$ for DMEM and $p < 0.04$ for RPMI.

This study examined the antioxidants EDTA, Se, and Zn because they are chelating and free radical scavenging agents that can reduce metal-catalyzed oxidation [36], particularly the oxidation of sulfhydryl (-SH) groups, as well as reduce protein oxidation and preserve protein integrity [37]. It was postulated that these actions would promote FGF-2 stability in DMEM and RPMI culture media. The concentration of antioxidants was thought to be appropriate for stabilizing proteins in aqueous solutions because they have antioxidant effects [19]. But, the data indicate that FGF-2 was unstable at 37˚C in both DMEM and RPMI,

and under the assessed conditions, none of them antioxidant was able to stabilize FGF-2 in either medium. The results indicate that the antioxidants tested did not stabilize FGF-2 at 37˚C, most likely due to the intrinsic structural instability of the FGF-2 under these conditions. This finding suggests that the thermal sensitivity of FGF-2 may limit its *in vitro* utility by accelerating the loss of its structural integrity.

Table 1. Percent recovery of FGF-2 in DMEM determined by RP-HPLC analysis. FGF-2 solutions (200 µg/mL) were prepared in DMEM containing 0.1 µM EDTA, Se, or Zn, each individually. For the control samples, 200 µg/mL FGF-2 was prepared in DMEM without antioxidants. Two sets of control vials were included: one stored at 2˚C - 8˚C, the other control and all test samples were incubated at 37˚C for designated time points. Then each sample and control solutions was analyzed independently by RP-HPLC, and the percent recovery of FGF-2 was calculated using Empower software. Freshly prepared FGF-2 in DMEM was defined as 100% recovery and served as the reference for determining the recovery of all other samples. Data represents mean values from three independent experiments.

Samples Name	% Recovery of FGF-2 in DMEM Medium						
	1 h	2 h	6 h	12 h	24 h	48 h	72 h
Freshly prepared FGF-2 in DMEM	100	100	100	100	100	100	100
FGF-2 in DMEM at 2˚C - 8˚C	112.1	107.9	105.5	101.6	95.8	85.4	0.0
FGF-2 in DMEM at 37˚C	63.0	53.7	29.5	10.5	0.0	13.4	0.0
FGF-2 in DMEM with EDTA at 37˚C	71.6	65.2	36.1	0.0	10.0	0.0	0.0
FGF-2 in DMEM with Se at 37˚C	105.5	76.8	63.0	17.3	19.1	0.0	0.0
FGF-2 in DMEM with Zn at 37˚C	103.0	81.8	41.6	13.8	16.8	0.0	0.0

Table 2. Percent recovery of FGF-2 in RPMI determined by RP-HPLC analysis. FGF-2 solutions (200 µg/mL) were prepared in RPMI supplemented individually with 0.1 µM EDTA, Se, or Zn. Control samples consisted of 200 µg/mL FGF-2 prepared in RPMI without antioxidants. Two control conditions were included: one set of vials stored at 2˚C - 8˚C and another set control and all test samples were incubated at 37˚C for designated time points. Then each sample and control solutions was analyzed independently by RP-HPLC, and FGF-2 percent recovery was quantified using Empower software. Freshly prepared FGF-2 in RPMI was defined as 100% recovery and served as the reference for calculating the recovery of all other samples. Data represents mean values from three independent experiments.

Samples Name	% Recovery of FGF-2 in RPMI medium						
	1 h	2 h	6 h	12 h	24 h	48 h	72 h
Freshly prepared FGF-2 in RPMI	100	100	100	100	100	100	100
FGF-2 in RPMI at 2˚C - 8˚C	103.0	82.0	80.4	88.1	81.3	75.5	0.0
FGF-2 in RPMI at 37˚C	76.2	75.2	58.8	0.0	0.0	0.0	0.0
FGF-2 in RPMI with EDTA at 37˚C	52.3	45.6	28.3	7.2	8.6	0.0	0.0
FGF-2 in RPMI with Se at 37˚C	75.1	66.1	32.4	10.6	11.0	0.0	0.0
FGF-2 in RPMI with Zn at 37˚C	77.1	75.8	0.0	12.5	11.7	0.0	0.0

The findings of this study indicate that antioxidants alone are not an effective strategy for stabilizing the structurally unstable FGF-2 at 37˚C [38] in either DMEM or RPMI media. The short half-life of FGF-2 in aqueous culture media may be attributed to its susceptibility to oxidation, aggregation, and proteolytic degradation [39-41] in DMEM and RPMI at 37˚C. Although this is not a typical method for FGF-2 stabilization, the prior study discovered that antioxidants [26] enhanced the stability of Epidermal Growth Factor (EGF) [26], which is different from FGF-2. Alternatively, complexing stabilizer like heparin can increase the stability of FGF-2, but heparin is widely recognized as an effective stabilizer for FGF-2 [11, 12], its utility is limited. At high concentrations, heparin can induce cytotoxicity, inhibit cell proliferation, and promote apoptosis in cultured cells, and its inherent anticoagulant activity limits its use as an inactive pharmaceutical antioxidant. However, the limitations of this study indicate that preserving FGF-2 stability in water based media such as DMEM and RPMI will require alternative strategies that are more convenient and effective than the antioxidants tested here.

4. DISCUSSION

Growth factors, like FGF-2, are essential signaling proteins that control cell division, proliferation, and survival. FGF-2 stability becomes essential for *in vitro* cell culture treatment. Although the stability of FGF-2 in solutions has been investigated [39, 40], it is still unclear how stable it is at 37˚C in commonly used cell culture media like DMEM and RPMI. Therefore, analyzing FGF-2 stability in cell culture media is very important. Three different antioxidants, including EDTA, Se, and Zn, were examined for their potential for stabilizing FGF-2 under physiological parameters to find components that can sustain FGF-2 stability throughout *in vitro* cell culture applications.

Antioxidants biocompatibility can be determined by using cell culture methods because the *in vitro* cytotoxic test [42] is a simple, repeatable, reproducible, appropriate, and suitable *in vitro* assay for evaluating fundamental biochemical properties. However, the colorimetric MTT test can be used to assess cytotoxicity and cell proliferation assays, as well as to calculate the percentage of live cells and metabolic activity [43]. After three days of incubation, the cytotoxicity of each antioxidant was evaluated by comparing the viability of the treated cells to that of the control cells, which had been grown to 100% viability. After three days of incubation, the viability of BALB/3T3 and NIH/3T3 cells was found to be more effectively maintained separately by EDTA, Se, and Zn up to 0.4 μM, indicating that these cell types are not sensitive to these antioxidants being studied.

When exposed to each antioxidant up to 5 μM, both BALB/3T3 and NIH/3T3 cells demonstrated an increase in growth rate; but microscopic observations showed that the cell morphology changed to a rounded shape at these higher concentrations of the antioxidants tested in this study. Therefore, the antioxidants were assessed at lowered concentrations. Neither cell line showed sensitivity to any of the antioxidants at concentrations as high as 0.4 μM for each, and their morphology was normal. Additionally, compared to those observed with Se and Zn, both cell lines demonstrated faster growth rates at the EDTA concentrations tested in this study, indicating that the cells are more tolerant of the EDTA concentrations used in this investigation. Furthermore, compared to EDTA and Se, the data indicates that after three days, both BALB/3T3 and NIH/3T3 cells showed lowered growth rates in response to Zn across the range of concentrations tested. This demonstrates that Zn toxicity varies with cell type, exposure time, and concentration [31]. Similarly, at all concentrations tested for Se, both cell lines showed reduced growth compared with EDTA. Since Se can probably regulate cell growth when added to cell culture medium [44], depending on the concentration, it may be toxic to cells [29]. Since Se serves as an antioxidant at lower concentrations and a pro-oxidant at higher concentrations, its results imply that Se is functioning as a pro-oxidant at higher concentrations [45]. However, the cytotoxicity assay results varied depending on the cell culture medium used and the procedures employed in the cytotoxicity investigations [46].

The results of the cytotoxicity assay showed that each antioxidant started to show cytotoxic effects at concentrations ranging from 0.4 μM and above after three days of exposure. For the proliferation tests, a concentration of 0.1 μM for each antioxidant (EDTA, Se, and Zn) was chosen to reduce cytotoxicity and

preserve normal cell morphology. To assess the proliferation of BALB/3T3 and NIH/3T3 cells separately, different concentrations of FGF-2 were tested for three days. The hypothesis was that by scavenging free radicals in the culture medium, the antioxidants at 0.1 μM could reduce oxidative stress and improve FGF-2 stability by keeping it in its reduced, physiologically active form. In comparison to the control under the same conditions, BALB/3T3 cells showed higher proliferation after three days of treatment. Although, compared to BALB/3T3 cells, NIH/3T3 cells exhibited lower proliferation under the same treatment conditions. These results imply that while FGF-2 and antioxidant containing supplements can lower apoptosis in NIH/3T3 cells, they are not enough to promote strong *in vitro* proliferation. This could indicate that for the best NIH/3T3 cell proliferation, different FGF-2 concentrations or specific cell culture conditions would be needed.

Since the FGF-2 samples were prepared in DMEM and RPMI media, it was crucial to make sure that no medium derived components affected FGF-2 detection during the analytical run. Additionally, RP HPLC analysis was carried out to support the hypothesis that stabilized FGF-2 could contribute to enhanced cell proliferation. FGF-2 was successfully separated from both DMEM and RPMI using the RP HPLC method, resulting in a distinct single peak. These results indicate that, under the experimental conditions used, the media components did not interfere with the analysis of FGF-2. Freshly prepared FGF-2 was considered as having 100% recovery and was used as the reference for calculating the recovery of all other FGF-2 samples.

The results of the RP-HPLC analysis showed that the stability of FGF-2 steadily decreased as incubation time in both DMEM and RPMI increased. When incubated at 2˚C - 8˚C, FGF-2 started to become less stable over time, and after 72 hours, it was completely degraded in both media types. After being incubated at 37˚C, FGF-2 rapidly lost stability. In DMEM, significant degradation occurred within 12 hours, whereas in RPMI, the FGF-2 was completely degraded after six hours. These results suggest that incubation at 37˚C in serum-free culture medium may increase the kinetic energy of FGF-2, leading to structural instability and denaturation. In addition, degradative processes may be accelerated under serum-free *in vitro* conditions, resulting in a more rapid loss of FGF-2 integrity in both DMEM and RPMI medium at 37˚C.

Similarly, when FGF-2 was prepared in DMEM or RPMI media containing individual antioxidants and incubated over multiple time points, the samples supplemented with EDTA showed detectable degradation within the first hour in both media. The extent of degradation increased progressively with longer incubation time. In DMEM, FGF-2 was completely lost after 12 h of incubation, whereas in RPMI, the FGF-2 was nearly fully degraded by 24 h. These observations demonstrate that EDTA does not provide stabilizing effects on FGF-2 in either DMEM or RPMI at 37˚C. When Se or Zn were added separately, FGF 2 was 100% detectable in DMEM at 37˚C for about an hour. However, as time went on, FGF-2 recovery began to decrease and was completely lost after 48 hours of incubation. However, in RPMI containing either Zn or Se, FGF-2 started to deteriorate after 1 hour under the same conditions. Furthermore, these findings show that FGF-2 in serum-free culture media is highly sensitive to 37˚C. In DMEM or RPMI conditions at 37˚C, FGF-2 was not stabilized by individual supplementation with 0.1 μM EDTA, Se, or Zn.

In addition, during the stability assessment, no FGF-2 degradation products such as monomers, covalent dimers, or high molecular-weight species were detected by RP-HPLC analysis. As expected, non-covalent aggregation is likely to increase when FGF-2 dissolution, ion complexation, salting-out effects, and charge neutrality converge near the protein's isoelectric pH. Under these conditions, the low pH and organic solvent composition of the mobile phase limit the ability to detect such noncovalent aggregates. However, neither DMEM nor RPMI media supplemented individually with EDTA, Se, or Zn showed any oxidized or cleaved FGF-2 species. These findings indicate that under *in vitro* DMEM or RPMI conditions at 37˚C, supplementation with 0.1 μM EDTA, Se, or Zn alone is insufficient to stabilize FGF-2. Additional bioassay studies may be required to confirm FGF-2 stability and activity in serum containing media. Serum proteins and endogenous growth factors may independently promote cell proliferation, as suggested by the proliferation experiments in which complete medium supplemented with antioxidants supported strong proliferation of cells. Components such as growth factors, amino acids, or serum albumin may further contribute to stabilizing FGF-2 and enhancing its biological activity.

5. CONCLUSION

BALB/3T3 and NIH/3T3 cells proliferated more when FGF-2 was supplemented separately with EDTA, Se, and Zn at 0.1 μM each in cell culture medium. Antioxidant supplementation did not increase FGF-2 stability, as determined according to reverse phase HPLC analysis of FGF-2 in serum free DMEM and RPMI. These results suggest that, independent of antioxidant addition, FGF-2 may be inherently unstable in DMEM and RPMI at 37˚C. Since serum contains albumin, endogenous growth factors, amino acids, and other carrier proteins that may directly support proliferation or help stabilize FGF-2, the increased proliferation seen in the cell based assays are most likely due to the presence of 10% serum. However, several findings regarding the stability of FGF-2 with antioxidants and cell toxicity have been documented in the literature, necessitating further investigation. Additionally, the findings indicate that variability in the observations may arise from differences in cell culture media, cell types, types and concentrations of antioxidants, as well as variations in laboratory procedures, all of which can affect the repeatability of these results.

AUTHORS CONTRIBUTIONS

A. S. Prakasha Gowda planned and organized the study, conducted the experimental work, analyzed the data, and prepared the manuscript. A.D. Schaefer was responsible for laboratory management and operational oversight throughout the study and manuscript review. All authors approved the final version for publication.

CONFLICTS OF INTEREST

The authors confirm that this article's content has no conflict of interest. This article does not contain any studies on 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.

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