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ORIGINAL ARTICLE

Evaluation of a Synthetic Polyethyleneimine Based Polymeric Vector for *ING4* Gene Delivery to MCF-7 Breast Cancer Cells

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ABSTRACT

Objectives: Breast cancer is the most common type of cancer among women and the second most common cause of death after lung cancer. The inhibitor of growth (ING) transcript levels are often suppressed in cancer cells, making it a promising candidate for cancer therapy. In this study, we aimed to formulate a polyplex that effectively carries and delivers pING4 to breast cancer cells.

Materials and Methods: Polyethyleneimine (PEI)-based non-viral vectors were synthesized and characterized for plasmid DNA delivery. Complexation was achieved *via* electrostatic interactions between the synthesized polymeric vectors and plasmid DNA. Characterization studies were conducted by testing Sodium dodecyl sulfate-induced complexation, Deoxyribonuclease I protection, and serum stability of the polyplexes. Subsequently, polyplexes were tested on MCF-7 cells for anticancer activity using the XTT cell viability assay. Western blot analysis was performed for the ING4 protein.

Results: Polyplexes carrying the *ING4* gene exhibited significantly lower cell viability than control polyplexes (p=0.0067). During the 5-day viability assay, the lowest cell viability was observed on day 4. Approximately 69.11±2.18% cell viability was observed with ING4 treatment and the control group showed no cell death on day 4 (101.53±5.06%). The prepared delivery systems did not show a toxic effect on MCF-7 cells treated alone. In addition, the MCF10A normal mammary cell line was used as a positive control. Western blotting was performed to confirm the overexpression of ING4 protein in the treatment groups. Unlike in the control groups, the overexpression of ING4 was clear in the wells of the treatment group.

Conclusion: Our findings suggest that *ING4* gene delivery using prepared PEI-based nonviral delivery systems is a promising approach for breast cancer treatment.

Keywords: *ING4*, polyethyleneimine, gene delivery, breast cancer

INTRODUCTION

Breast cancer is the most common cancer in women globally, and the number of cases is still increasing, according to the World Health Organization. In particular, patients with metastasis have a very low survival rate.[1] Currently, there are several methods exist for the treatment of breast cancer: chemotherapy, immunotherapy, hormone therapy, surgery, and radiotherapy. Surgery is the first choice, mostly because it makes sense to remove most of the tumor tissue. Chemotherapy and radiotherapy applications follow surgery for clearing remnant cancer cells.[2,3] However, treatment using these methods is not recommended. Furthermore, all of these methods have a low level of patient compliance and cause a decrease in the patients' quality of life.[3] However, advanced technology has brought novel techniques to the clinic. Gene therapy is one such therapy.

The basic definition of gene therapy is the transfer of DNA to the patient to cure diseases. Gene therapy can be used to trigger

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the expression of desired proteins within cells. Viral and non-viral vectors can be used for this purpose. The physiological nature of viruses allows them to pack and efficiently deliver specific genes to target cells. To date, most gene therapy trials have been conducted using viral vectors for this reason. However, there are serious concerns regarding viral gene therapy, including immunogenicity and insertional mutagenesis. Both have a high risk of death. In addition, large-scale virus production is not very cost-effective. All of the aforementioned disadvantages can be discarded using non-viral vectors. Non-viral vectors are capable of carrying genes, protecting them from several nucleases, and delivering the genes to desired locations effectively.[4-7]

Peptides, lipids, and polymers can be used to formulate non-viral vectors. All non-viral vector types have their advantages. However, polymer-based vectors can be considered one step ahead because of their high transfection ability.[8] In addition, polymers can be synthesized desirably because they can be modified. Polyethyleneimine (PEI) has been used extensively for designing nonviral vectors.[6,7] PEI is a commercially available synthetic polymer with a repeating unit composed of the amine group and two carbons aliphatic CH_2CH_2 spacer. PEI exhibits remarkable efficacy in the formation of polyanionic complexes with plasmid DNA. Polyplex formation between PEI and pDNA occurs *via* electrostatic interactions, and PEI can protect the pDNA from nuclease degradations.[9] Moreover, PEI is recognized for its ability to induce the "proton sponge" effect owing to its robust buffering capacity under acidic pH conditions.[6] High cytotoxicity is the only major disadvantage of PEI-based vectors.[7] However, there are different types of PEI available (linear or branched, different molecular weights, *etc.*), and their toxic effects can be reduced with modifications. In recent years, advancements in non-viral gene delivery have led to a variety of methods and materials. PEI stands out as a gold standard, ensuring superior transfection efficacy due to its effective DNA binding, protection, and high endosomolytic competence, particularly through lPEI/pDNA polyplexes, which enhance DNA translocation to the nucleus and exhibit improved cell viability and transfection efficiency.[10]

The Inhibitor of Growth (ING) family genes were identified in 1996. INGs are evolutionarily conserved proteins located in the nucleus.[11] ING4 is a constituent of a tumor suppressor protein family comprising five members (ING1-5). ING4, with a molecular weight of 29 kDa, functions as a type II tumor suppressor protein and holds crucial significance as an integral member of the ING protein family. It has two Nuclear Localization Signals and is located in the cell nucleus. It exerts tumor suppressor activity by regulating angiogenesis, metastasis, invasion, cell cycle arrest, and apoptosis. Additionally, ING4 plays a role in chromatin remodeling. It contains a plant homeodomain finger motif that facilitates chromatin-mediated gene regulation.[12,13] ING4 is also linked with p53, NF-B, and HIF-1B and regulates their activities. ING4 exhibits predominant loss or downregulation at the RNA level across various cancer types. Furthermore, multiple studies reported the loss of ING4 protein expression in breast cancer.[11-17] The *ING4* gene has been used as a biomarker for breast cancer.[18] Unfortunately, the mechanism underlying the loss of the *ING4* gene remains unclear.[13] The ability of ING4 to inhibit neoangiogenesis and cell migration resulted in its label as "gatekeeper".[19] It has been reported that pING4 (a pDNA that encodes ING4 protein) can suppress tumor growth and with that exhibit prolonged survival time.[20,21]

The utilization of PEI-based polymeric vectors for plasmid DNA delivery has emerged as a pivotal advancement in gene therapy research. These vectors, owing to their cationic nature and excellent condensation properties, play a critical role in enhancing the stability and protection of DNA cargos during transportation. In the context of *ING4* gene delivery, the use of PEI-based polymeric vectors not only ensures efficient and targeted transfer of the therapeutic gene into cancer cells but also offers a promising avenue for the development of precise and potent treatments for breast cancer and other malignancies. In recent years, the exploration of innovative gene delivery strategies has become paramount in cancer research. This study focused on the delivery of the *ING4* gene to breast cancer cells through PEI-based polymeric vectors, indicating the beginning of a new phase in the creation of reliable and efficient therapeutic interventions. We aimed to formulate a polyplex that effectively carries and delivers pING4 to breast cancer cells.

MATERIALS AND METHODS

Materials

The pcDNA3-ING4 plasmid was obtained from Addgene as a bacterial stab (USA). Additionally, pcDNA3 plasmid DNA was generously provided as a gift by Prof. Dr. Zeki Topçu from the Pharmaceutical Biotechnology Department, Faculty of Pharmacy, Ege University, İzmir, Türkiye. Both plasmids were expanded and purified using an Invitrogen maxipen DNA proliferation kit (USA). For the cell-based experiments, Dulbecco's Modified Eagle's Medium (DMEM) F12 medium, fetal bovine serum (FBS), and XTT cell proliferation kits were obtained from Biological Industries (USA). Phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (USA). RIPA Lysis and Extraction Buffer were obtained from ThermoFisher Scientific (USA). MCF10A and MCF-7 cell lines were obtained from the American Type Culture Collection (USA). Deoxyribonuclease I (DNase I) was obtained from New England Biolabs (USA). The 1.2-kDa branched polyethylenimine was procured from Polysciences Inc. (Warrington, PA, USA). Linoleyl chloride (LA) was obtained from NU-CHEK PREP (Elysian, MN, USA). Propionic acid, acryloyl chloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), chloroform, and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis and characterization of PEI-based polymeric vectors

Hydrophobically modified PEI1.2tLA6 polymers were synthesized via N-acylation using carboxyl end-capped aliphatic lipids.[22] The synthesis process is explained in detail in the aforementioned paper.[22] In summary, LA and mercaptopropionic acid (MPA) were individually dissolved in trifluoroacetic acid.

The MPA solution was cautiously added dropwise to the LA solution under light-protected conditions. The resulting product was the carboxyl end-capped LA, hereafter referred to as tLA. PEI-tLA was obtained by grafting tLA to branched PEI1.2. The grafting process was carried out with EDC/NHS activation. Obtained PEI1.2tLA was characterized by H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA).

Polyplex formation

Polyplexes were formulated at room temperature by combining an aqueous solution of pDNA (0.4 µg/µL) with the pre-synthesized polymer solution. The polymer/DNA ratio was adjusted to 5 (*w/w*). The polyplex suspension was left at room temperature (25 °C) for 30 minutes before transfection.

Sodium Dodecyl Sulfate (SDS)-induced DNA release (decomplexation)

Agarose gel electrophoresis was used to assess the release profile of DNA from polyplexes.[23] Polyplexes were incubated with SDS for 5 min at 25 °C. The samples were then loaded onto a 1% agarose gel for electrophoresis. Final SDS ratios between 1% and 8 were assessed to determine the optimal release. After subjecting the samples to electrophoresis at 90 V for 1 hour, visualization was conducted under ultraviolet (UV) light following a 10-minute staining period with ethidium bromide (EtBr).

DNase I protection

1 U of DNase I enzyme was used for each 2.5 µg DNA in this study.[24] DNase I was added after polyplex formation. Tubes containing DNase I were incubated at 37 °C in a water bath for 30 minutes. Following the incubation period, SDS, the quantity previously determined (as described in the preceding section), was introduced to facilitate DNA release from the polyplexes. The resulting samples were then loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100 volts. After 10 minutes of EtBr staining, the gel was photographed under UV light.

In vitro serum stability assessment

The stability of DNA integrity can be tested in vitro using FBS containing various nucleases.[25] Serum stability testing was performed to determine the degree of protection from the enzymes found in serum.[26] Resistance of DNA within polyplexes to serum degradation was assessed in a serum stability study at 37 °C using both 10% and 50% FBS to mimic *in vitro* blood conditions. The experiments were performed at distinct time intervals of 1, 6, and 24 hours. After each incubation period, a release solution consisting of SDS at the rate determined in the complexation study and Proteinase K at a concentration of 2 mg/mL was added to the samples. The DNA integrity was subsequently analyzed using agarose gel electrophoresis under the aforementioned conditions.

Cell culture

XTT was performed to determine cell proliferation.[27] MCF10A and MCF-7 cells were used in cell culture studies. The cells were cultured in DMEM F12 medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. The cell culture was maintained in a humidified atmosphere with 5% CO_2 at 37 °C throughout the study period.

Cells were seeded into 48-well plates at a density of 25,000 cells/well and incubated for 24 hours before transfection. Subsequently, polyplexes were added at a volume of 20 µl/well. The cells were rinsed with PBS following the incubation period. Cell viability was assessed using the XTT reagent according to the manufacturer's instructions. The untreated cells served as the baseline cells with 100% viability. All experimental treatments were performed in triplicate for statistical rigor. Cell viability in treated wells was expressed as a percentage and calculated using the following formula:

$$\text{Cell viability (\%)} = [(Abs_{sample}/Abs_{control})\text{-}Abs_{blank}] \times 100$$

Protein extraction and western blot analysis

Following the transfection process, protein extraction was performed for western blot analysis.[28,29] Polyacrylamide gels and buffers were prepared according to the protocols of Sambrook et al.[30]. The cells were harvested and lysed using modified RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the bicinchoninic acid assay (Sigma, UK). Subsequently, SDS-polyacrylamide gel electrophoresis and western blot analyses were conducted under standard conditions using 50 µg of protein lysate per Lane. The proteins were separated on 12% gels and transferred to polyvinylidene fluoride (PVDF) membranes (Sigma, UK) using a wet transfer blotter.

To prevent non-specific binding, the PVDF membrane was blocked with 5% dry milk in tris-buffered-saline [(TBS-T) TBS-T solution containing 0.1% tween 20]. Primary antibody incubation was performed using an ING4 polyclonal antibody from Elabscience (E-AB-33309), followed by Horseradish peroxidase (HRP)-conjugated secondary antibody incubation, both conducted in TBS-T containing 0.5% dry milk either at room temperature for 1 hour or at 4 °C overnight.

For visualization, the membranes were developed using the chemiluminescent HRP substrate ECL reagent at a 1:1 ratio (Thermo Fisher Scientific, USA) for 4 minutes and then photographed using an image analyzer equipped with a charge-coupled device camera. Subsequently, a densitometric band intensity analysis was performed using ImageJ software.

Statistical analysis

Statistical analysis was conducted using the GraphPad Prism 6.0 software. The cell culture results were analyzed using Student's t-test, and a p value less than 0.05 was considered statistically significant.

RESULTS

SDS-induced polyplex decomplexation

It is important that polymers can successfully release DNA as well as form polyplexes. The SDS-induced release study was conducted to observe the ability of the polyplexes. Another aim of this study was to determine the optimal SDS

amount for releasing DNA, which will be necessary in future studies. SDS concentrations of 1-8% were tested. Figure 1 illustrates successful DNA release by polyplexes at all SDS concentrations. Optimal release was achieved at 5% SDS (Lane 7). Furthermore, it can be concluded that the DNA release is intact since the band luminosityis close to that of control DNA (Lane 1).

DNase I protection ability of polyplexes

SDS-induced decomplexation showed that the polymers can release intact DNA. Nevertheless, it is important that a delivery system can protect its cargo from DNase I. Effective gene expression requires protection of the DNA inserted into the cell from nuclease degradation.[31] Figure 2 shows a gel image of a DNase I protection study.

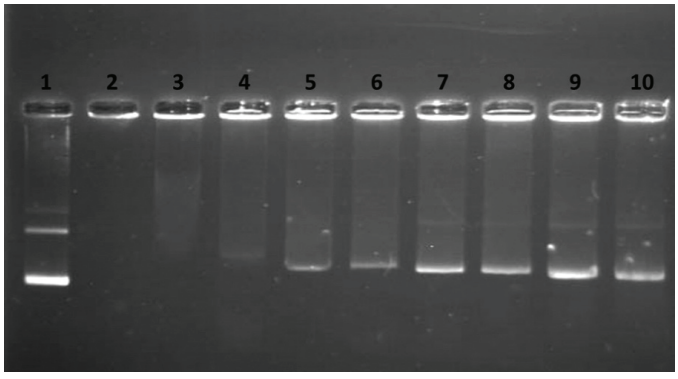

Figure 1. Agarose gel image of the decomplexation study. Lane 1: Naked DNA as control, Lane 2: Polyplex control, Lane 3-10: SDS% with pDNA respectively; 1-8%

SDS: Sodium dodecyl sulfate

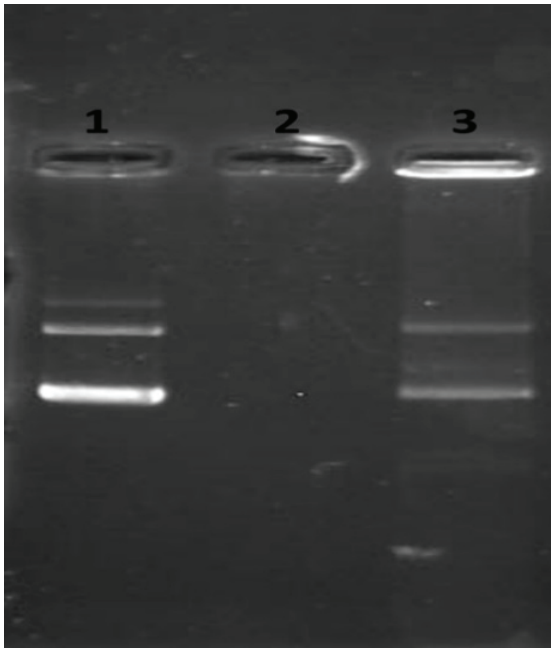

Figure 2. Agarose gel image of a DNase I protection study. Lane 1: Naked DNA control, Lane 2: Naked DNA + DNase I, Lane 3: Polyplex + DNase I + SDS 5%

SDS: Sodium dodecyl sulfate

The presence of serum proteins poses a significant challenge to DNA integrity. Abundant nuclease enzymes in serum can cleave the phosphodiester bonds between sugar and phosphate moieties of DNA, leading to its degradation. In addition, serum opsonin causes opsonization resulting in phagocytosis.[25] Therefore, it is important to protect the cargo DNA from serum proteins. DNA digestion by serum nucleases is shown in Figure 3A. Lane 1 shows the naked DNA as a positive control. Digested DNA at different time intervals can be observed in Lane 2, Lane 3, and Lane 4. Figure 3B-D shows the serum protection ability of the synthesized polymer at three intervals of time (1, 6, and 24 hours) as the bands are visible. Lane 1 is the positive control, as mentioned before. Lanes 2 and 3 represent 10% and 50% FBS protection, respectively.

Cell culture

The cell growth curves demonstrated that proliferation was inhibited in the pcDNA3-ING4-transfected group in a time-dependent manner (Figure 4). There was a significant difference between the pcDNA3 control and pcDNA3-ING4 treatment group in the days following 2nd day ($p < 0.05$). Maximum inhibition was spotted on day 4 as 32.42%.

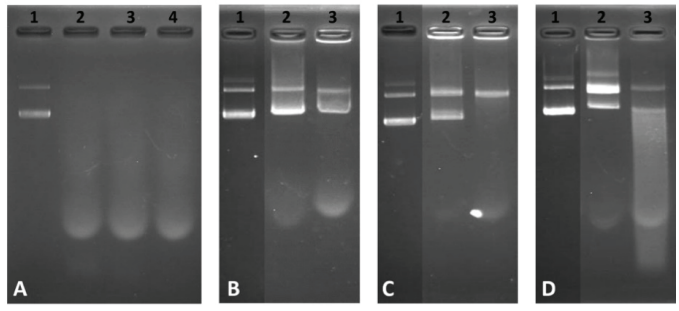

Figure 3. Agarose gel images of serum stability measurements. A: DNA without polymer (Lane 1: Naked DNA control, Lane 2: DNA + FBS - 1 hour, Lane 3: DNA + FBS - 6 hours, Lane 4: DNA + FBS - 24 hours), B-D: 1-6-24 hours (Lane 1: Naked DNA control, Lane 2: Polyplex + FBS 10%, Lane 3: Polyplex + FBS 50%)

FBS: Fetal bovine serum

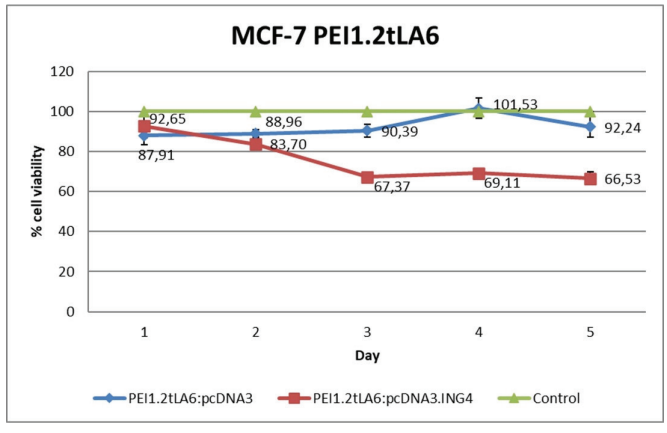

Figure 4. MCF-7 breast cancer cell line 5-day cytotoxicity study

MCF10A cell line is used as a normal human mammary cell line for positive control against the MCF7 cancerous cell line.[32] On day 4, the cell lines treated with pcDNA3 and pcDNA3-ING4 showed no statistically significant difference ($p > 0.05$), as illustrated in Figure 5.

Overexpression of ING4 in MCF-7 cells via polyplex-induced transfection

Western blotting was used to assess ING4 overexpression following polyplex transfection. Increased overexpression was detected in pcDNA3-ING4-transfected cells compared with the control groups based on the densitometric band intensity analysis, suggesting that the synthesized polymer successfully transfected the ING4 plasmid. ING4 expression levels were normalized against β-actin expression. Figure 5 shows significantly elevated ING4 expression.

DISCUSSION

The successful release of DNA from polyplexes, especially at the optimal 5% SDS concentration determined in our study, not only validates the efficiency of our designed polymeric vectors but also ensures the integrity of the released genetic material.

Figure 2 presents a gel image from the DNase I protection study, demonstrating in Lane 3 that the polyplexes effectively protect DNA from DNase I digestion and enable its successful release. Lane 2 shows the absence of discernible bands, indicating the digestion of plasmid DNA by DNase I in the absence of a delivery system. The DNase I protection study without a doubt demonstrates the polymers' ability to shield encapsulated DNA from enzymatic degradation. The polyplexes in Lane 3 effectively protected and released DNA, in stark contrast to Lane 2, where naked DNA succumbs to DNase I digestion.

PEI-based vectors can protect nucleic acids at high (50%) serum concentrations.[33] Furthermore, it is known that PEI exerts successful endosomal escape ability via the proton sponge effect.[6,34] According to the findings of this study, in contrast to naked plasmid DNA, which undergoes rapid degradation by serum nucleases, our designed polymeric vector exhibits remarkable stability, effectively protecting the encapsulated DNA cargo from enzymatic degradation.

A considerable number of *in vitro* studies focused on breast cancer consisting of MCF7 cells considering their estrogen-responsive characteristics. This specialty of MCF7 cells makes them a useful model for breast cancer biology studies.[35] The ability to inhibit proliferation in cancer cells, while not affecting normal cells is a critical step toward developing targeted and effective cancer treatments. Furthermore, western blot results align with those of previous studies in the field, corroborating the importance of these proteins in cancer biology.[29]

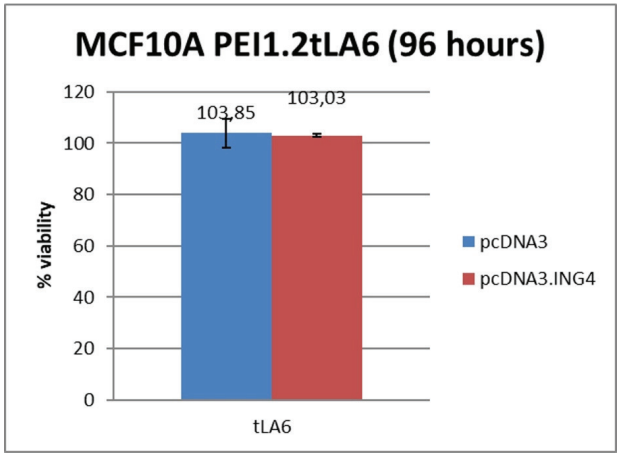

Figure 5. Cytotoxicity of the MCF10A cell line cytotoxicity study as positive control

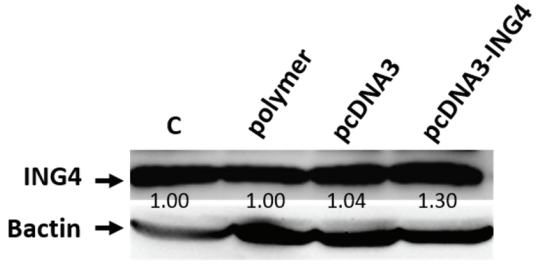

Figure 6. Protein expression of ING4 with western blot analysis

CONCLUSION

In conclusion, a PEI-based non-viral vector was synthesized and complexed with a plasmid that encodes the ING4 protein. It is also an important feature that the formulation can protect DNA from serum proteins.[36] Notably, our polyplexes exhibited potent cytotoxicity against cancer cells while maintaining non-toxicity in control DNA. Western blotting confirmed the presence of the ING4 protein, affirming the efficacy of our approach. These findings strongly support the potential of our formulation as a promising candidate for non-viral gene therapy in breast cancer treatment, emphasizing its viability for further preclinical and clinical investigations.

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Ethics

Ethics Committee Approval: There is no requirement for ethical approval.

Informed Consent: There is no requirement for informed consent to be obtained.

Authorship Contributions

Concept: U.K., Design: U.K., A.G.K., H.U., Data Collection or Processing: U.K., E.I., Analysis or Interpretation: U.K., R.B.KC., E.I., A.G.K., H.U., Literature Search: U.K., Writing: U.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study received no financial support.

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