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RESEARCH ARTICLE

Vitamin D_3-Loaded Nanoemulsions as a Potential Drug Delivery System for Autistic Children: Formulation Development, Safety, and Pharmacokinetic Studies

Marwa Hasanein Asfour[1] · Sameh Hosam Abd El-Alim[1] · Ahmed Alaa Kassem[1] · Abeer Salama[2] · Amr Sobhi Gouda[3] · Walaa Samy Nazim[3] · Neveen Hassan Nashaat[4] · Maha Hemimi[4] · Nagwa Abdel Meguid[4]

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Abstract

The aim of the current study is the development of a vitamin D_3 (VD3)-loaded nanoemulsion (NE) formulation to improve VD3 oral bioavailability for management of vitamin D inadequacy in autistic children. Eight NE formulations were prepared by high-speed homogenization followed by ultrasonication. Four vegetable oils were employed along with two concentrations of Span 20 as the emulsifier. Glycerol, fructose, and mango flavor were included as viscosity modifier, sweetening, and flavoring agents, respectively. The prepared VD3-loaded NE formulations exhibited high drug content (> 98%), droplet size (DS) ranging from 61.15 to 129.8 nm with narrow size distribution, zeta potential values between − 9.83 and − 19.22 mV, and acceptable pH values (4.59–5.89). Storage stability showed that NE formulations underwent coalescence and phase separation during 6 months at room temperature, whereas at refrigerated conditions, formulations showed slight creaming. The optimum formulation (VD3-NE6) revealed a non-significant DS growth at refrigerated conditions and spherical morphology under transmission electron microscopy. VD3-NE6 did not produce any toxic effects to rats treated orally for 3 months, where normal blood picture and kidney and liver functions were observed compared to control rats. Also, serum calcium, oxidative stress, and apoptosis biomarkers remained within normal levels, indicating the safety of the optimum formulation. Furthermore, evaluation of VD3-NE6 oral bioavailability depicted a significant increase in AUC_{0-72} and C_{max} with decreased T_{max} compared to plain VD3. The optimum formulation demonstrated improved stability, safety, and oral bioavailability indicating the potential for successful management of vitamin D deficiency in autistic children.

Keywords autism · nanoemulsion · pharmacokinetics · toxicity · vitamin D_3

Introduction

Several studies reported that patients with autism spectrum disorder (ASD) suffer from nutritional and metabolic anomalies such as mitochondrial dysfunction, methylation impairment and oxidative stress [1]. The etiology of ASD is still uncertain in the majority of cases; nevertheless, combinations of various environmental and genetic aspects are potentially contributing factors [2]. Evidentially, mineral and vitamin supplementation could assist these vital physiologic processes [1]. As one important factor of the proposed pathogenesis of autism, nutrient insufficiencies are of specific concern. Moreover, the characteristics and behavior of ASD and autistic particular involvements could escalate the hazard of sub-optimal nutrition [3]. Autistic children are generally selective with restricted food preferences based on sensory modalities such as taste, smell, and texture [4]. A recent study highlighted the contribution of palatability to food acceptance among those children [5]. Moreover, the gastrointestinal abnormalities in autism impair the absorption of micronutrients [6]. Consequently, the threat of nutrient insufficiency is becoming of scientific importance [7]. Nutrient supplementation can be highly effective with no

✉ Sameh Hosam Abd El-Alim
samehhosam@yahoo.com

[1] Pharmaceutical Technology Department, National Research Centre, El-Buhouth St., Dokki 12622, Cairo, Egypt

[2] Pharmacology Department, National Research Centre, El-Buhouth St., Dokki 12622, Cairo, Egypt

[3] Biochemical Genetics Department, National Research Centre, El-Buhouth St., Dokki 12622, Cairo, Egypt

[4] Research On Children With Special Needs Department, National Research Centre, El-Buhouth St., Dokki 12622, Cairo, Egypt

adverse effects, as no molecules foreign to the body are used [8].

Vitamin D is a fat-soluble micronutrient which does a vital task in skeletal functions, e.g., bone health and calcium absorption in addition to non-skeletal functions like inhibiting diabetes, cardiovascular diseases, and cancers [9, 10]. The deficiency of vitamin D is considered one of the most widespread micronutrient malnutrition disorders [11, 12]. Its inadequate intake could lead to osteoporosis, rickets, calcium–phosphorus imbalance, parathyroid imbalance, and diabetes [13]. Chemically, vitamin D has two major forms, namely, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) [14]. In humans, vitamin D_3 (VD3) has more potency compared to vitamin D_2 owing to its greater efficiency to raise blood serum levels of the bioactive metabolites of vitamin D [15]. Upon exposure to UV radiation, VD3 is naturally synthesized in human skin cells. However, due to low exposure to sunlight and/or poor diet in many countries, vitamin D deficiency is still widespread [9, 16].

Recently, vitamin D has gained attention in research concerning the field of psychiatry [3]. Current studies have revealed a significantly lower vitamin D level in autistic children compared to healthy ones [17–20]. Accordingly, the supplementation of vitamin D for autistic children is necessary [21]. VD3 is highly sensitive to environmental stresses and is liable to oxidation which leads to losing its physiological benefits and functionality [14]. It also exhibits poor water solubility and oral bioavailability (44.8%) [22]. Being a fat-soluble vitamin, it is commonly absorbed with fatty acids and fats at particular sites in the small intestine via both active and passive transport [23]. Thus, the use of lipid-based drug delivery systems for encapsulation of VD3 would essentially lead to an enhancement of its bioavailability [24, 25]. Moreover, encapsulating VD3 in these delivery systems will also enable its release in a controlled manner and allow the administration of optimal doses, thus avoiding potential side effects of hypervitaminosis syndrome [13].

Nanoparticulate systems loaded with drugs, also called nanodrugs or nanomedicines, have shown remarkable outcomes linked to the absence of collateral toxicities and improved drug efficacy [26]. Nanoemulsion (NE) is a lipid-based drug delivery system that mainly consists of oily phase, aqueous phase, and surfactant [27]. It is a suitable way for nanodispersion of lipophilic bioactives in aqueous environments to be further used in foods and pharmaceuticals [28, 29]. NEs are biocompatible, biodegradable, simple to fabricate, and employed as platforms for hydrophobic therapeutic agents that undergo hydrolysis [30]. Compared to conventional emulsions, NE droplets are characterized by their small size that potentially enhance their stability to coalescence, gravitational separation, and flocculation [31]. NEs reveal outstanding drug release profile because of the big interfacial area [32]. It has been also proposed that the smaller the NE droplet size, the higher the bioavailability of encapsulated lipophilic compounds [33–35]. They may also be administered through different routes depending on their intended application [36].

Accordingly, this work aims to formulate and evaluate NEs for the encapsulation of VD3 for the management of vitamin D deficiency in ASD children. Safe ingredients were employed for the preparation of NEs such as vegetable oils and non-ionic surfactants. Safety, on basis of acute and chronic toxicity, and pharmacokinetic studies on experimental animals were performed in comparison with plain VD3. Clinical studies on ASD children are underway.

Materials and Methods

Materials

Vitamin D_3 (colecalciferol; VD3) was bought from Sigma-Aldrich Co., USA. 25-Hydroxyvitamin D_3 [25(OH)D3] ($\geq 97\%$) and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) were supplied by Santa Cruz Biotechnology, Inc., USA. Span 20 (Sp20) (extra pure) was purchased from Loba Chemie Pvt. Ltd., India. Glycerol ($\geq 99\%$) was obtained from Fisher Scientific Co., UK. Fructose (99%) was procured from Winlab Ltd., UK. Almond, pumpkin, olive, and wheat germ oils were bought from the specific unit of extracting oils at the National Research Centre (NRC), Cairo, Egypt. The cold press method was used for oil extraction. Mango flavor was purchased from Kamena Co., Egypt. Phosphotungstic acid hydrate was supplied by Fluka analytical, USA. All other chemicals and reagents were of analytical grade. Creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), calcium (Ca), and reduced malondialdehyde (MDA) kits were supplied by Biodiagnostic Co., Egypt. Enzyme-linked immunosorbent assay (ELISA) kit of caspase was procured from Sunlong Biotech Co., Ltd., China.

Methods

Preparation of VD3-Loaded NEs

Based on the preliminary studies, Sp20, glycerol, fructose, and mango flavor were selected as the emulsifier, viscosity modifier, sweetening, and flavoring agents, respectively. The preparation of VD3-loaded NE took place by the method previously reported with some modifications [27]. In brief, VD3 (0.0015%, w/v) was dissolved in the vegetable oil (almond, pumpkin, olive or wheat germ oil) (5%, v/v) along with Sp20

(2 or 3%, v/v) and mixed forming the oily phase. Glycerol (10%, v/v), fructose (30%, w/v), mango flavor (0.2%, v/v), and double distilled water were mixed to form the aqueous phase. The oily phase was added slowly to the aqueous phase while mixing on the magnetic stirrer (1500 rpm) for 5 min. Subsequently, the formed mixture was passed across a high-speed homogenizer (SilentCrusher M, Heidolph Instruments GmbH & Co. KG, Germany) at 20,000 rpm for 10 min followed by sonication employing a bath sonicator (Elmasonic S 40 H, Elma Schmidbauer GmbH, Germany) for 15 min. The resulting NE was kept in a refrigerator for upcoming examinations. The composition of the developed VD3-loaded NE formulations is illustrated in Table I.

Characterization of VD3-Loaded NEs

Estimation of VD3 Content VD3 content in the prepared VD3-loaded NE formulations was determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS). Briefly, 1 ml of the formulation was appropriately diluted by methanol and sonicated in the bath sonicator for 20 min. Afterwards, the solvent was evaporated under a nitrogen stream. The derivatization reagent, namely, 150 µl of PTAD in acetonitrile (1 mg/ml), was added to the residue, vortex-mixed, and kept at room temperature for 1 h for reaction completion. The sample was evaporated under a nitrogen stream followed by reconstitution in 40% acetonitrile (400 µl) to be analyzed [37]. The assessment was carried out in triplicate. VD3 content was computed as follows:

$$\text{VD3 content}(\%) = \frac{\text{Estimated amount of VD3}}{\text{Initial amount of VD3 added}} \times 100$$

LC–MS/MS was performed on a Waters Xevo TQD triple quadrupole tandem mass spectrometer coupled to Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system, Waters Co., USA. Chromatographic separation was conducted using Waters Acquity UPLC BEH C_{18} column (2.1 × 100 mm, 1.9 µm) maintained at 40°C. Acetonitrile (B) and 0.1% formic acid in water (A) were used as mobile phases [37]. Gradient elution was carried out at a flow rate of 0.2 ml/min. The gradient program was as follows: 0 to 3 min 40% B, 9 to 10.5 min 60% B, 11 to 14 min 90% B, and 18 to 25 min 40% B. The injection volume was 5 µl. Mass spectrum (MS) analysis was performed employing a positive mode electrospray ionization method. A multiple reaction mode was used for quantitation with parent daughter mass transition 560.4 < 298.1, cone voltage 30 V, and collision energy 20 V.

Droplet Size, Polydispersity Index, and Zeta Potential Measurements The droplet size (DS), polydispersity index (PDI), and zeta potential (ZP) of the developed VD3-loaded NE formulations were investigated via dynamic light scattering using a Zetasizer (Nano Series ZS90, Malvern Instruments Ltd., UK) at 25°C. Formulations were appropriately diluted by double distilled water to achieve a suitable scattering intensity. The width of the size distribution was assessed by PDI determination. A high PDI value points to a heterogeneous size distribution, while a low PDI value indicates a greater homogeneity [38].

Employing the same apparatus, ZP of the developed VD3-loaded NE formulations was determined via examining their electrophoretic mobility in an electrical field. All measurements were assessed using 3 independent formulation samples.

pH Measurement The pH of the prepared VD3-loaded NE formulations was determined using a standard digital pH meter (3510 pH meter, Jenway Co., UK) at 25°C.

Storage Stability Study

The storage stability was inspected by storing the prepared VD3-loaded NE formulations at room (25 ± 2°C) and refrigeration (4 ± 2°C) temperatures for a period of 6 months. Three independent samples from each formulation were

Table I Composition of the Prepared 0.0015% Vitamin D_3-Loaded Nanoemulsion Formulations

Formulation code	Almond oil (%)	Pumpkin oil (%)	Olive oil (%)	Wheat germ oil (%)	Sp20 (%)	Glycerol (%)	Fructose (%)	Mango flavor (%)	Water (%)
VD3-NE1	5	–	–	–	2	10	30	0.2	52.8
VD3-NE2	5	–	–	–	3	10	30	0.2	51.8
VD3-NE3	–	5	–	–	2	10	30	0.2	52.8
VD3-NE4	–	5	–	–	3	10	30	0.2	51.8
VD3-NE5	–	–	5	–	2	10	30	0.2	52.8
VD3-NE6	–	–	5	–	3	10	30	0.2	51.8
VD3-NE7	–	–	–	5	2	10	30	0.2	52.8
VD3-NE8	–	–	–	5	3	10	30	0.2	51.8

kept in firmly sealed glass containers and stored under dark conditions. At 2, 4, and 6 months, samples were visually inspected for any signs of creaming, coalescence, phase separation, and/or precipitation. At the end of the study (6 months), VD3 content, DS, PDI, ZP, and pH of the samples were determined.

Transmission Electron Microscopy (TEM)

Morphological examination of the optimum VD3-loaded NE formulation was performed via TEM (JEM-2100, JEOL Co., Japan). The optimum formulation was appropriately diluted by double distilled water. A drop of the formulation sample was added on a carbon-coated Cu grid and left for 10 min to dry completely. Subsequently, phosphotungstic acid solution (1%, w/v) was added to the sample for staining and air-dried for 10 min at 25°C. Surface characteristics and shape of the optimum NE formulation was then assessed at suitable magnifications.

In vivo Studies

Animals Healthy male and female Wistar albino rats (120–140 g) were employed for the current studies. Rats were procured from the central animal house at the NRC, Cairo, Egypt. Animals were kept in well-ventilated boxes (22 ± 2°C) on a 12-h light and dark cycle. Rats were fed pelleted food and tap water *ad libitum*. Rats were humanely treated, and the experiment protocols took place following the ethical guidelines regarding care and use of experimental animals approved by the Medical Research Ethics Committee at the NRC (Reg. No. 19/233).

Acute Toxicity Study Forty eight Wistar albino rats (24 males and 24 females) were classified into four groups of twelve rats each. Groups were divided in two subgroups containing six rats each, representing both sexes (male and female), and received treatments as follows: group 1 comprised normal control male and female rats receiving a single oral dose of normal saline. Group 2 consisted of male and female rats given a single oral dose of plain VD3 at a dose of 1800 IU/kg, i.e., 45 µg/kg. Male and female rats in groups 3 and 4 were given a single oral dose of the optimum VD3-loaded NE formulation and drug-free NE formulation, respectively, at a dose equivalent to 1800 IU/kg, i.e., 45 µg/kg of VD3. Noteworthy, the administered dose of VD3 was calculated as ten times the recommended daily dose in adults, i.e., 20,000 IU [39], according to conversion tables by Paget and Barnes [40]. Rats were observed daily for a period of 2 weeks. They were checked for occurrence of morbidity and mortality as well as for any change in skin and fur appearance, respiratory rates, central nervous system disturbances (tremors, convulsions, lethargy, sleep disturbance, coma, etc.), and behavioral effects.

Chronic Toxicity Study Chronic toxicity was carried out according to the World Health Organization research guidelines [41]. Eighty Wistar albino rats (40 males and 40 females) were allocated into four groups of twenty rats each. Groups were divided in two subgroups containing ten rats each of both sexes as follows: group 1 consisted of normal control male and female rats given an oral dose of normal saline once daily for a period of three months. Group 2 comprised male and female rats receiving an oral dose of plain VD3 at a dose of 180 IU/kg, i.e., 4.5 µg/kg once daily for 3 months. Male and female rats in groups 3 and 4 were given a daily oral dose of the optimum VD3-loaded NE formulation and drug-free NE formulation, respectively, at a dose equivalent to 180 IU/kg, i.e., 4.5 µg/kg of VD3 once daily for 3 months. The administered dose of VD3 was calculated according to conversion tables by Paget and Barnes [40] based on a recommended adult daily dose of 2000 IU [39].

At the end of the study (3 months), animals were anesthetized with pentobarbital sodium, and blood samples were taken from the retro-orbital plexus. A complete blood count (CBC) was carried out.

Blood samples were collected then centrifuged at 4°C using a cooling centrifuge (2k15, Sigma Laborzentrifugen GmbH, Germany) at 3000 rpm for 15 min to separate the serum. Creatinine and BUN were determined according to the methods of Bartels *et al.* [42] and Fawcett and Scott [43], respectively. Liver function enzyme activities (AST and ALT) were estimated in rat serum according to the methods of Reitman and Frankel [44], whereas ALP was assessed according to Belfield and Goldberg [45]. Serum Ca, MDA, and caspase levels were also determined according to Gindler and King [46], Ohkawa *et al.* [47], and Mohamed *et al.* [48], respectively.

Pharmacokinetic Study Twelve male Wistar albino rats were allocated into two groups of six rats each. Group 1 received a single oral dose of plain VD3 at a dose of 180 IU/kg, i.e., 4.5 µg/kg. Group 2 was given a single oral dose of the optimum VD3-loaded NE formulation at a dose equivalent to 180 IU/kg, i.e., 4.5 µg/kg. The administered dose of VD3 is equivalent to the dose used for the chronic toxicity study. At determined time intervals (up to 72 h), blood samples were withdrawn from the retro-orbital venous plexus using heparinized capillary tubes. Blood samples were centrifuged at 3000 rpm for 10 min to separate plasma and kept at − 80°C until LC–MS/MS analysis. The pharmacokinetic parameters for orally administered VD3 were calculated based on a non-compartmental model [49]. The determined parameters were the area under the plasma drug concentration *vs.* time

curve from 0 to 72 h (AUC_{0-72}), maximum plasma drug concentration (C_{max}), and time needed to reach maximum plasma drug concentration (T_{max}). Parameters were calculated directly from the individual plasma drug concentration *vs.* time profiles. The data procured from pharmacokinetic parameters were analyzed statistically using PKSolver, an add-in program for pharmacodynamic, and pharmacokinetic data analysis in Microsoft® Excel [50].

After thawing at room temperature, 2 ml of acetonitrile was added to 1 ml of plasma for protein precipitation. Samples were vortex-mixed followed by centrifugation for 10 min at 3000 rpm. The supernatants were transferred to glass tubes, and the volume was reduced to 1 ml by evaporation under nitrogen stream. Vitamin D metabolites were extracted from the remaining solution by liquid–liquid extraction via adding 5 ml of ethyl acetate followed by vigorous shaking for 10 min and then centrifuged for 5 min at 1000 rpm. The upper organic layer was transferred to new glass tubes, and the solvent was completely evaporated under nitrogen stream [37]. Derivatization took place by adding 150 µl of PTAD to the residue and continued as previously discussed in the "Estimation of VD3 Content" section.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted employing one way analysis of variance (ANOVA) followed by Tukey's post hoc for means comparison using GraphPad Prism software, version 5 (GraphPad Inc., San Diego, USA). The difference was considered significant at $p < 0.05$.

Results and Discussion

Preparation of VD3-Loaded NEs

All eight NE formulations showed a homogenous white milky appearance. No signs of coalescence or phase separation were reported after 24 h of preparation. The selection of ingredients and their concentrations was mainly focused on safety, taste acceptability, and NE stability. Almond, olive, pumpkin, and wheat germ oils were selected for the present investigation. Vegetable oils are known for their safety on oral administration. A preliminary study was performed to assess the most suitable ingredients and concentrations for the formation of NEs using the four investigated oils. The examined surfactants were Tween 20, Tween 80, and Sp20. NEs prepared employing Tween 20 and Tween 80 revealed cracking after 24 h irrespective of the oil type. On the contrary, NEs prepared employing Sp20 exhibited no signs of separation or cracking with the four oils investigated after 24 h. Using surfactants with hydrophilic–lipophilic balance (HLB) values close to those required for oils results in the formation of more stable emulsions [51, 52]. The required HLB value of vegetable oils is generally around 7 [52]. The required HLB value for the four investigated oils ranges from 6 to 8, making Sp20 (HLB = 8.6) a more appropriate surfactant for NE preparation, compared to Tween 20 and Tween 80 (HLB = 16.72 and 15, respectively). Thus, Sp20 was selected for use in the current study. Sp20 is a non-ionic surfactant commonly employed in cosmetics and pharmaceutical preparations as well as food products. It is considered as a nonirritant and nontoxic ingredient [53, 54]. Glycerol is widely utilized as a sweetening agent, solvent, viscosity-increasing agent, and preservative in oral solutions [54]. Furthermore, the addition of small quantities of glycerol was reported to notably augment the storage stability of oil in water emulsions [55, 56]. Fructose is commonly employed in solutions, syrups, and tablets as a sweetening and flavoring agent. The sweetness-response profile of fructose is recognized more quickly in the mouth compared to that of dextrose and sucrose. This might account for the capability of fructose to improve fruit flavors and hide particular unpleasant mineral or vitamin "off-flavors" [54]. VD3 was incorporated in the NE formulations at a concentration of 15 µg/ml, i.e., 600 IU/ml, which is equivalent to the recommended daily oral dose for children from 1 to 10 years old [39].

Characterization of VD3-Loaded NEs

Estimation of VD3 Content

All developed VD3-loaded NE formulations exhibited high drug content (> 98%), thus confirming that VD3 is completely incorporated in NE formulations (Table II). NEs are found to provide elevated encapsulation efficiency and enhanced drug stability [27].

Droplet Size, Polydispersity Index, and Zeta Potential Measurements

Measurement of DS is a significant parameter for assessing the quality of the prepared NE formulations [57]. Smaller DS results in greater interfacial area for drug absorption leading to quicker absorption and enhanced bioavailability [58]. The DS of the investigated VD3-loaded NE formulations ranged from 61.15 to 129.80 nm indicating that NE droplets were in the nanometric range (Table II). This relatively small DS range is possibly ascribed to the stabilization of oil droplets due to the existence of the surfactant molecules at the

oil–water interface [58]. The surfactant might contribute to the condensation and stabilization of the interfacial film, leading to lesser DS [58, 59]. The results also show that formulations containing a higher amount of Sp20 (3%, v/v) exhibited smaller DS compared to their counterparts comprising 2%. This decrease in DS upon increasing the amount of surfactant was previously reported [59–61]. Increasing the amount of surfactant efficiently stabilized the droplets via forming a steric barrier on their surface, hence guarding smaller droplets and hindering them to coalesce into bigger ones [27, 62].

PDI reflects the homogeneity of size distribution. The results reveal that PDI values of the tested NE formulations ranged from 0.339 to 0.436 (i.e., < 0.5), which indicates uniformity of DS distribution and good homogeneity [63]. This comes in accordance with previous reports [63, 64]. The results also show that an increase in PDI was observed as the surfactant concentration increased from 2 to 3% (Table II). It has been reported that the concentration of surfactant required to stabilize NEs is considerably high due to the large surface to volume ratio of droplet interfaces. Since many surfactants form micelles at such high concentrations, the continuous phase of NEs usually contains micelles as a reservoir of surfactant for coating the droplet interfaces [65]. Accordingly, it is common to employ surfactants at concentrations higher than their critical micelle concentrations for NE preparation as previously reported [66, 67]. This could plausibly explain the observed increase in the PDI as the surfactant concentration increased.

ZP assessment provides an indication of the electrostatic repulsion between oil droplets. An increase in electrostatic repulsion between droplets decreases the chance of their coalescence into larger globules [57, 68]. The assessed VD3-loaded NE formulations showed moderate negative ZP values ranging from − 9.83 to − 19.22 mV (Table II). The negative charge shown by NE formulations is possibly attributed to the presence of Sp20. Previous reports showed that NE systems, prepared employing non-ionic surfactants, exhibited moderately negative ZP values [69, 70]. This was attributed to adsorption of anionic species from the water such as hydroxyl ions to the droplet surfaces [70]. This would explain the higher |ZP| values recorded for NE formulations consisting of Sp20 (3%, v/v) compared to their corresponding ones containing 2%. Another possible explanation could be the presence of glycerol. Emulsion systems containing glycerol were reported to exhibit negatively charged ZP that increased with the increase of glycerol concentration. This may be explained by its negatively charged hydroxyl side groups [55].

pH Measurement

The pH values of the prepared VD3-loaded NE formulations ranged from 4.59 to 5.89 (Table II). Liquid products possessing a slightly acidic pH have better palatability [71].

Storage Stability Study

NEs are kinetically stable and thermodynamically unstable systems, which will eventually separate into different phases. Hence, they are less sensitive to physical and chemical changes, whereas increasing the temperature promotes the destabilization of NEs because of the change in the dispersed phase solubility and diffusivity [72]. Accordingly, it is essential to assess the stability of NEs under different storage conditions. NEs should maintain their physical and chemical stability during the product's shelf life [73, 74]. Table III shows the visual inspection of the prepared VD3-loaded NE formulations throughout storage at room (25 ± 2°C) and refrigeration (4 ± 2°C) temperatures for 6 months. Formulation samples stored at room temperature demonstrated coalescence or phase separation after 2, 4, and 6 months. On the other hand, samples stored at refrigeration temperature for 2 months remained stable without any signs of drug precipitation, creaming, coalescence, cracking, or phase separation. Nevertheless, after 4 and 6 months, samples revealed slight creaming, which was resolved after mild shaking (Table III). This observation comes in accordance with a previous study by Desai *et al.* where all formulations exhibited phase separation at room temperature after 30 days, while no phase separation was observed at refrigerated temperature [75]. Also,

Table II Characterization Parameters of the Prepared 0.0015% Vitamin D_3-Loaded Nanoemulsion Formulations ($n = 3$)

Formulation code	VD3 content (%) ± SD	DS (nm) ± SD	PDI ± SD	ZP (mV) ± SD	pH ± SD
VD3-NE1	98.72 ± 0.35	96.92 ± 8.53	0.339 ± 0.029	− 11.37 ± 1.52	5.27 ± 0.02
VD3-NE2	98.46 ± 0.39	92.88 ± 7.99	0.373 ± 0.031	− 15.17 ± 1.74	5.60 ± 0.03
VD3-NE3	98.27 ± 0.28	129.80 ± 11.86	0.368 ± 0.042	− 9.83 ± 1.03	4.59 ± 0.02
VD3-NE4	98.16 ± 0.43	109.81 ± 10.43	0.436 ± 0.056	− 12.29 ± 1.33	4.76 ± 0.01
VD3-NE5	98.64 ± 0.56	89.58 ± 8.24	0.359 ± 0.037	− 16.40 ± 1.68	5.60 ± 0.04
VD3-NE6	99.36 ± 0.29	61.15 ± 6.76	0.370 ± 0.039	− 17.99 ± 1.95	5.75 ± 0.03
VD3-NE7	98.77 ± 0.32	88.86 ± 9.11	0.396 ± 0.045	− 16.30 ± 1.72	5.71 ± 0.02
VD3-NE8	99.69 ± 0.47	78.53 ± 8.04	0.419 ± 0.054	− 19.22 ± 2.09	5.89 ± 0.04

Table III Visual Inspection of the Prepared 0.0015% Vitamin D_3-Loaded Nanoemulsion Stored at Room (25 ± 2°C) and Refrigeration (4 ± 2°C) Temperatures for 6 Months

Formulation code	Temperature (°C)	2 months	4 months	6 months
VD3-NE1	Room	Coalescence	Phase separation	Phase separation
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE2	Room	Coalescence	Phase separation	Phase separation
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE3	Room	Coalescence	Phase separation	Phase separation
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE4	Room	Coalescence	Phase separation	Phase separation
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE5	Room	Coalescence	Coalescence	Coalescence
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE6	Room	Coalescence	Coalescence	Coalescence
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE7	Room	Coalescence	Coalescence	Coalescence
	Refrigeration	Homogenous NE	Creaming	Creaming
VD3-NE8	Room	Coalescence	Coalescence	Coalescence
	Refrigeration	Homogenous NE	Creaming	Creaming

cracking was observed in NE systems stored for 50 days at 37°C by Agnish *et al.*, whereas stable systems were observed upon storage at refrigerated temperature [76]. Instability of NE systems at higher temperatures could be attributed to the presence of oxidation products of the oil. Alterations in the characteristics of NE systems stored at room temperature were previously reported by Maruno. This could be justified by a possible chemical instability due to degradation of formulation compounds caused by hydrolysis of the oil, accompanied by greater solubility of specific oil components in the continuous phase [77]. Increased solubility of oil components in the continuous phase would greatly affect physical stability of NE systems, leading to an increase in the rate of Ostwald ripening [78]. These findings indicate that the developed NE formulations as a drug delivery system should be kept at refrigerated conditions throughout the shelf life. Gravitational separation is the process in which NE droplets move upward (creaming) or downward (sedimentation) as a result of having a lower or higher density compared to the surrounding medium. Because most liquid oils have lesser density than water, they will have the tendency to migrate upwards, whereas water will move downwards [79]. Creaming is considered insignificant in NEs until the DS increases to the size of a few microns because of flocculation, coalescence, and Ostwald ripening [72].

At the end of the storage period (6 months), NE formulations stored at refrigeration temperature were checked for their VD3 content, DS, PDI, ZP, and pH. Compared to zero time samples, the change in drug content was non-significant ($p > 0.05$). Conversely, the results depicted that a significant ($p < 0.05$) increase in DS was observed compared to zero time samples except for VD3-NE6, where a non-significant difference ($p > 0.05$) was revealed (Tables II and IV). Furthermore, formulations prepared employing Sp20 (2%, v/v) showed a significant ($p < 0.05$) increase in DS compared to their counterparts (3%). This finding comes in agreement with previous reports [75, 80]. This could be attributed to the stabilizing effect produced by a higher surfactant concentration. The hydrophobic groups of the surfactant are adsorbed onto the droplet surface, whereas the hydrophilic groups extend into the aqueous phase in the form of a coil, acting as an effective barrier against aggregation [81]. Generally, because of the emulsifier layer adsorbed on the NE droplets, steric interactions will increase the repulsive force which will in turn stabilize the NEs against coalescence and flocculation [72].

Compared to zero time samples, all investigated NE formulations revealed a relative increase in PDI values after 6 months (Tables II and IV). In fact, PDI is potentially correlated to the Ostwald ripening, where a higher PDI indicates a higher difference in chemical potential between droplets [72]. The increase in both DS and PDI of NE systems upon storage was previously reported by Compolo *et al*. Both parameters increased in all tested formulations in correlation with time of storage up to 28 weeks [69].

On the other hand, a relative decrease in both |ZP| and pH values was observed in all examined NE formulations compared to zero time samples (Tables II and IV). This comes in accordance with previous reports [69, 82–84]. This change in ZP values could be attributed to the formation of oxidation products, which cause a reduction in pH of the medium [85]. It was previously reported that NEs, at neutral pH values, exhibit higher stability, whereas at lower pH values, droplets increase in size [86]. Electrostatic

Table IV Characterization Parameters of the Prepared 0.0015% Vitamin D_3-Loaded Nanoemulsion Stored at Refrigeration Temperature (4 ± 2°C) for 6 Months ($n = 3$)

Formulation code	VD3 content (%) ± SD	DS (nm) ± SD	PDI ± SD	ZP (mV) ± SD	pH ± SD
VD3-NE1	98.44 ± 0.32	294.20 ± 31.58	0.448 ± 0.049	− 11.09 ± 1.12	5.01 ± 0.01
VD3-NE2	98.17 ± 0.36	184.22 ± 29.74	0.437 ± 0.042	− 13.46 ± 1.19	5.29 ± 0.02
VD3-NE3	98.12 ± 0.24	481.21 ± 85.34	0.408 ± 0.038	− 5.98 ± 0.81	4.31 ± 0.02
VD3-NE4	98.05 ± 0.38	177.53 ± 16.28	0.480 ± 0.041	− 11.10 ± 1.14	4.49 ± 0.03
VD3-NE5	98.49 ± 0.42	208.60 ± 22.63	0.363 ± 0.033	− 8.16 ± 0.93	5.42 ± 0.01
VD3-NE6	99.22 ± 0.23	65.81 ± 5.41	0.401 ± 0.043	− 17.16 ± 1.63	5.69 ± 0.02
VD3-NE7	98.61 ± 0.29	308.60 ± 26.72	0.426 ± 0.044	− 8.10 ± 0.96	5.37 ± 0.01
VD3-NE8	98.94 ± 0.40	89.62 ± 8.44	0.457 ± 0.047	− 16.64 ± 1.75	5.52 ± 0.03

repulsion between droplets is diminished at these low pH values as a result of adsorption of hydrogen ions (H^+) to the surface of the droplets. This will result in a lower negative charge and aggregation in media of high acidity [87]. It should be noted that the stability of NE systems is owed mainly to steric repulsion rather than electrostatic repulsion [69]. In view of the aforementioned results, it can be obviously seen that NE formulations prepared using olive oil showed a relatively smaller change in DS, ZP, and pH values after 6 month. This could be attributed to the fact that olive oil is less susceptible to oxidation compared to the other vegetable oils because it contains less polyunsaturated fatty acids [52, 88]. Olive oil is characterized by the presence of high fraction of monounsaturated fatty acids [89, 90]. In fact, the higher the degree of unsaturation, the higher the oxidation rate [52]. This is because the hydrogen atom attached to the carbon of the double bond is removed with ease, yielding alkyl radicals [91].

Based on the preceding results, VD3-NE6, exhibiting the smallest DS, optimum PDI, ZP, pH values, and the highest storage stability, was chosen for upcoming examinations.

Transmission Electron Microscopy

Figure 1 shows the TEM micrograph of VD3-NE6. NE droplets appeared as well dispersed, dark stained, and spherical in shape. No aggregation or coalescence was observed. The DS of the NE is in accordance with the hydrodynamic diameter obtained via the dynamic light scattering measurements (the "Droplet Size, Polydispersity Index, and Zeta Potential Measurements" section).

In vivo Studies

Acute Toxicity Study

Daily inspection for 2 weeks of both male and female rats given a single dose of plain VD3, VD3-NE6, and drug-free NE6 formulations did not show any signs of toxicity in both sexes, i.e., no mortality, hair loss, diarrhea, change in skin appearance, or behavioral abnormalities.

Chronic Toxicity Study

The CBC picture of both male and female rats receiving a daily oral dose of plain VD3, VD3-NE6, and drug-free NE6 formulations for 3 months did not demonstrate any significant changes in hematological parameters ($p > 0.05$) in comparison with the normal control group (Table V).

Oral administration of plain VD3, VD3-NE6, and drug-free NE6 formulations daily for 3 months did not produce any significant changes ($p > 0.05$) in kidney (creatinine and BUN) and liver (ALT, AST, and ALP) function parameters, oxidative stress (MDA), and apoptosis (caspase) biomarkers as well as serum Ca of both male and female rats compared to the normal control group (Table VI).

In light of the acute and chronic toxicity studies, VD3-NE6 is considered a safe formulation.

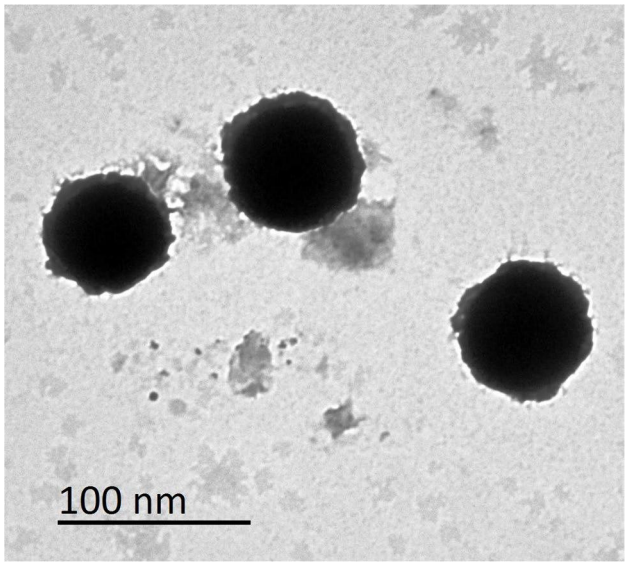

Fig. 1 Transmission electron microscope micrograph of the optimum vitamin D_3-loaded nanoemulsion formulation stained with phosphotungstic acid (1%, w/v)

Table V Complete Blood Count Picture of Male and Female Rats Receiving a Daily Oral Dose of Different Treatments (4.5 µg/kg) for 3 Months ($n=10$)

Parameter	Group							
	Male rats				Female rats			
	Normal Control	Plain VD3	Drug-free NE6	VD3-NE6	Normal Control	Plain VD3	Drug-free NE6	VD3-NE6
HB (g/dl) ± SD	12.44 ± 1.35	14.08 ± 1.01	14.16 ± 0.21	12.74 ± 1.72	12.60 ± 1.89	13.24 ± 0.93	13.04 ± 1.59	13.06 ± 0.47
RBCs (10^{12}/l) ± SD	6.04 ± 0.32	8.04 ± 0.65	7.46 ± 0.16	7.10 ± 1.00	5.91 ± 1.55	6.95 ± 0.93	5.92 ± 1.51	6.72 ± 0.73
Hematocrit (%) ± SD	35.08 ± 2.38	37.52 ± 1.58	39.12 ± 1.54	38.78 ± 2.72	36.12 ± 5.11	37.80 ± 3.18	33.60 ± 3.67	34.28 ± 2.65
MCV (fl) ± SD	56.68 ± 0.91	51.50 ± 1.56	54.68 ± 1.31	50.74 ± 2.40	52.52 ± 6.27	52.96 ± 2.37	52.16 ± 3.46	50.70 ± 0.85
MCH (pg) ± SD	19.70 ± 0.57	17.66 ± 0.78	19.24 ± 0.28	17.90 ± 0.79	19.08 ± 1.29	18.14 ± 1.49	18.12 ± 0.65	17.94 ± 0.27
MCHC (g/dl) ± SD	34.22 ± 0.51	35.64 ± 0.66	35.50 ± 1.01	35.26 ± 0.83	34.20 ± 1.30	34.58 ± 2.03	33.96 ± 0.93	33.48 ± 1.55
Platelets (10^9/l) ± SD	640.80 ± 68.01	600.60 ± 43.18	690.40 ± 57.27	665.40 ± 65.29	784.20 ± 131.18	740.20 ± 73.03	749.20 ± 53.79	771.20 ± 118.83
WBCs (10^9/l) ± SD	12.68 ± 1.54	13.86 ± 0.73	12.62 ± 1.02	14.40 ± 1.80	12.44 ± 1.71	10.28 ± 0.42	12.80 ± 4.04	14.52 ± 3.16

Table VI Biochemical Parameters of Male and Female Rats Given a Daily Oral Dose of Different Treatments (4.5 µg/kg) for 3 Months ($n=10$)

Parameter	Group							
	Male rats				Female rats			
	Normal Control	Plain VD3	Drug-free NE6	VD3-NE6	Normal Control	Plain VD3	Drug-free NE6	VD3-NE6
Creatinine (mg/dl) ± SD	0.83 ± 0.04	0.81 ± 0.02	0.85 ± 0.02	0.81 ± 0.01	0.91 ± 0.10	0.84 ± 0.03	0.87 ± 0.04	0.86 ± 0.09
BUN (mg/dl) ± SD	2.71 ± 0.40	2.84 ± 0.19	2.47 ± 0.13	2.67 ± 0.43	2.36 ± 0.28	2.27 ± 0.21	2.49 ± 0.14	2.51 ± 0.12
ALT (U/L) ± SD	10.86 ± 0.76	10.66 ± 0.68	10.66 ± 0.79	11.06 ± 1.02	11.48 ± 0.81	10.58 ± 0.12	11.70 ± 0.54	11.30 ± 0.67
AST (U/L) ± SD	23.99 ± 2.02	26.18 ± 1.05	22.09 ± 1.72	24.04 ± 1.88	25.74 ± 1.49	21.64 ± 1.15	24.79 ± 3.34	23.49 ± 2.34
ALP (IU/L) ± SD	86.70 ± 9.24	80.12 ± 6.45	82.59 ± 5.96	87.69 ± 6.98	90.01 ± 7.34	76.66 ± 3.03	89.02 ± 6.06	82.10 ± 6.40
Ca (mg/dl) ± SD	10.49 ± 0.22	10.40 ± 0.51	10.15 ± 0.16	10.57 ± 0.43	11.09 ± 0.13	10.76 ± 0.18	11.02 ± 0.05	11.07 ± 0.54
MDA (nmol/ml) ± SD	182.00 ± 3.19	170.38 ± 34.67	156.00 ± 14.28	172.00 ± 24.83	183.88 ± 24.88	173.75 ± 23.23	157.5 ± 37.97	162.76 ± 28.99
Caspase (pg/ml) ± SD	3.82 ± 0.40	3.34 ± 0.53	3.63 ± 0.74	3.56 ± 0.42	3.61 ± 0.55	3.05 ± 0.34	3.87 ± 0.36	3.54 ± 0.49

Pharmacokinetic Study

The current study was conducted to explore the ability of VD3-NE6 to improve the gastrointestinal absorption of VD3 after oral administration. Determination of serum 25(OH) D3 metabolite is usually accepted as the most reliable indicator for evaluation of the individual VD3 blood level [49]. The plasma 25(OH)D3 concentration *vs.* time curve for both plain VD3 and VD3-NE6 is presented in Fig. 2. An obvious improvement of pharmacokinetic parameters (AUC, C_{max}, and T_{max}) in rats given a single oral dose of VD3-NE6 compared to plain VD3 group was observed. Determination of the pharmacokinetic parameters is important for comparative bioavailability studies. AUC represents the extent of drug absorption, while C_{max} and T_{max} are essential parameters of the plasma drug level profile, which are characteristic parameters of the drug formulation [92]. Evaluation of the pharmacokinetic parameters showed a significant increase ($p<0.05$) in AUC_{0-72} (316.18 ng.h/ml) and C_{max} (8.01 ng/ml) of rats receiving VD3-NE6 compared to plain VD3 (173.05 ng.h/ml and 4.69 ng/ml) by 1.8-fold and 1.7-fold, respectively (Table VII). Additionally, VD3-NE6 group revealed a clear decrease in T_{max} (4 h) compared to plain VD3 group (7.33 h). These results indicate the superiority of VD3-NE6 in increasing the absorption of VD3. This comes in agreement with a previous study [49].

VD3 is known to have poor water solubility and oral bioavailability (less than 50%) [22]. It is generally absorbed at definite sites in the small intestine together with fatty acids and fats by means of active and passive transport [23]. NEs have been previously reported to increase oral bioavailability of lipophilic drugs [75, 93]. The surfactant component of the NE may increase permeability through direct partitioning into the cell membrane and disrupting the structural organization of the lipid bilayer resulting in penetration improvement [75]. Furthermore, the lipophilic components in the NE have strong affinity with the lymphatic system and thereby may increase drug transport

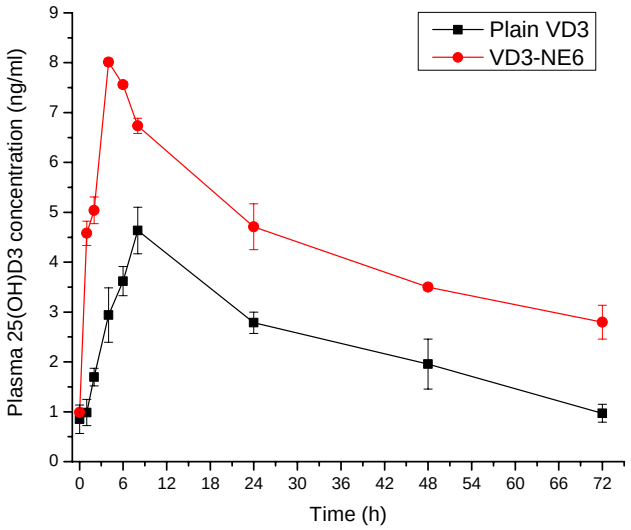

Fig. 2 Plasma 25-hydroxyvitamin D_3 concentration–time profile after administration of a single oral dose of the optimum vitamin D_3-loaded nanoemulsion formulation and plain vitamin D_3 (4.5 µg/kg) to male rats ($n=6$). Data were presented as mean ± SD

Table VII Pharmacokinetic Parameters After Administration of a Single Oral Dose of the Optimum Vitamin D_3-Loaded Nanoemulsion Formulation and Plain Vitamin D_3 (4.5 µg/kg) to Male Rats ($n=6$)

Group	AUC_{0-72} (ng.h/ml) ± SD	C_{max} (ng/ml) ± SD	T_{max} (h) ± SD
VD3-NE6	316.18 ± 12.44	8.01 ± 0.06	4.00 ± 0.00
Plain VD3	173.05 ± 23.69	4.69 ± 0.92	7.33 ± 1.15

through the intestinal lymphatic pathway. Having the drug in a solubilized form within the NE might also be an important factor [93]. Additionally, VD3-NE6 exhibited a comparatively small DS (< 100 nm) which plays an important role in NE stability and a crucial step in the course of improving drug bioavailability [94, 95]. Smaller DS results in a greater interfacial area for drug absorption leading to quicker absorption and enhanced bioavailability [58]. Moreover, nanocarrier systems are reported to extend residence time on mucosal membranes with permeation enhancing properties leading to an increased drug uptake [96]. Other reports demonstrated that the nanometric size of emulsion droplets enhanced intestinal permeation of therapeutic agents [97, 98]. Accordingly, the developed VD3-NE6 formulation would potentially result in improved VD3 oral bioavailability [24, 25].

In light of the obtained results, VD3-NE6 would be a good candidate for VD3 supplementation for autistic children. It has been previously reported that vitamin D levels are significantly lower in children with ASD compared to healthy controls, which could classify them as vitamin D inadequate. Accordingly, it is suggested that vitamin D insufficiency may play a role in the etiology of autism [20]. Previous studies have assessed the effect of VD3 supplementation in children with ASD. However, inconsistent results were reported as VD3 supplementation significantly improved the outcome of some children with ASD [99]. Conversly, other reports revealed limited and inconsistent effects of VD3 supplementation [100]. The clinical study to be undertaken by the authors aims to further investigate the subject through making use of the potential enhancement of VD3 bioavailability of VD3-NE6. Supplementation with VD3-NE6 will be provided to children with ASD and compared to a marketed product containing an equivalent dose of VD3. VD3-NE6 is expected to overcome poor intestinal absorption peculiar to autistic children. This will potentially lead to improving vitamin D blood levels and better management of the disease.

Conclusion

Successful preparation of eight VD3-loaded NE formulations was achieved employing four different vegetable oils (5%), Sp20 (2 and 3%), glycerol (10%), fructose (30%), and mango flavor (0.2%). All formulations have efficiently encapsulated VD3 (> 98%) with nanometric DS (61.15–129.8 nm), narrow size distributions (< 0.5), moderately negative ZP (− 9.83 to − 19.22 mV), and acceptable pH values (4.59–5.89). The storage stability study revealed that all formulations exhibited coalescence and phase separation at room temperature after 6 months. At refrigerated conditions, all formulations showed no signs of physical instability for 2 months and only slight creaming after 4 and 6 months. The optimum formulation, VD3-NE6, comprising olive oil (5%) and Sp20 (3%), exhibited a non-significant DS growth upon storage at refrigerated temperature. It appeared as spherical droplets under TEM with no aggregations. Toxicological assessments in male and female rats for 3 months proved the safety of the optimum formulation. Compared to normal control animals, no significant changes to blood picture, kidney, and liver functions as well as serum Ca, oxidative stress, and apoptosis biomarkers were reported. Moreover, a single oral dose of VD3-NE6 given to male rats demonstrated improved pharmacokinetic parameters in comparison with plain VD3, indicating a better drug absorption. Hence, VD3-NE6 depicted a great potential for oral delivery of VD3 with enhanced stability, safety, and bioavailability. Meanwhile, further studies on the clinical management of vitamin D inadequacy in children with ASD are underway.

Author Contribution Marwa Hasanein Asfour: conceptualization, methodology, investigation, data curation, writing original draft, and writing review and editing.

Sameh Hosam Abd El-Alim: conceptualization, methodology, data curation, investigation, writing original draft, and writing review and editing.

Ahmed Alaa Kassem: conceptualization, methodology, data curation, investigation, writing original draft, and writing review and editing.

Abeer Salama: methodology, investigation, data curation, and writing original draft.

Amr Sobhi Gouda: methodology, validation, and investigation.

Walaa Samy Nazim: methodology, validation, and investigation.

Neveen Hassan Nashaat: resources and software.

Maha Hemimi: resources and software.

Nagwa Abdel Meguid: conceptualization, project administration, and funding acquisition.

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Data Availability All data generated or analysed during this study will be available on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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