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Post-operative supplementation with vitamin D after mucogingival surgery significantly enhances autophagy and improves life quality following feline chronic gingivostomatitis

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ABSTRACT

Oral mucositis is a complex inflammatory and ulcerative condition frequently associated with a heightened risk of infections, malnutrition and diminished quality of life in both humans and animals. Despite the availability of various preventive and therapeutic interventions, their overall efficacy remains unclear. Considering that vitamin D exhibits pronounced anti-inflammatory properties by modulating autophagic pathways via activation of the vitamin D receptor (VDR), the present study aims to determine whether supplementation with vitamin D after the mucogingival replacement surgery (MGRS) would effectively enhance autophagy, and therefore, protect the integrity of mucosal lining in cases of severe oral mucositis. Adult domestic cats suffered from feline chronic gingivostomatitis and undergoing MGRS were used in this study. After MGRS, experimental cats were orally administered either fat-soluble or water-soluble vitamin D at a dose of 200 ng/kg twice daily for 6 weeks. Quantitative analysis revealed that in cats with oral mucositis and received MGRS, post-operative supplementation of both types of vitamin D greatly improved the quality of life and increased the anti-inflammatory reactivity. Moreover, both types of vitamin D considerably enhanced the expression of VDR and light chain 3B (LC3B, a biochemical marker for autophagy) within the affected tissues, with the most notable change observed in cats that received fat-soluble vitamin D. Based on these findings, incorporating vitamin D into the post-operative care regimens may enhance the therapeutic efficacy of surgical interventions targeting severe mucosal injury. This strategy may also hold a novel promise for improving the overall management of oral mucositis and associated complications.

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1. Introduction

Oral mucositis is an inflammatory and ulcerative lesion of the oral mucosal lining that frequently occurs in humans and animals [1–3]. Among the small animals, cats are more susceptible to severe oral mucositis (such as feline chronic gingivostomatitis, FCGS) due to their heightened immune response to oral antigens like plaque bacteria or viruses [4,5]. Severe FCGS would cause moderate-to-severe oral pain, decreased or absent appetite, malnutrition and reduced socialization, which significantly impairs the quality of life of affected cats [6]. Clinical management goals for FCGS generally focus on decreasing or eliminating antigenic stimulation, with tooth extraction or removing affected tissues serving as a primary strategy to reduce inflammation and improve the quality of life for cats [6,7]. However, although surgical intervention could effectively reduce a portion of chronic inflammatory burden, there are nearly one-third of affected cats did not respond to surgical treatments like partial/fully tooth extractions or gingivectomy [7–9]. This condition underscores the importance of extended medical management with antimicrobial or anti-inflammatory medications following surgical intervention to achieve significant improvement or a complete cure of FCGS [6,9,10].

Vitamin D is an essential organic compound best known for its critical roles in regulating calcium phosphate metabolism and bone remodelling [11]. Over the past few decades, the discovery of vitamin D receptors (VDR) in various cells and organs has revealed that vitamin D plays a role not only in regulating calcium homoeostasis but also in modulating immune responses through its potent anti-

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inflammatory and antimicrobial activities [12]. Previous studies have indicated that supplementation with vitamin D could prevent the occurrence of radiation-induced oral mucositis and exert a positive effect on periodontal health [13–15]. Pharmacological reports also demonstrated that treatment with vitamin D may exhibit a chemo-preventive and therapeutic potential against oral squamous cell carcinoma as well as enhance osseointegration that subsequently increases the success rate of dental implants [16–19]. It is indicated that the advanced effects of vitamin D against oral pathologies may closely linked to its capacity to regulate autophagy [20,21]. By activating VDR-mediated signalling pathways, vitamin D can modulate the expression of genes involved in autophagy, promoting the clearance of damaged cells, reducing inflammatory responses and thereby enhancing the processes of mucosal healing and maintaining the overall integrity of the oral cavity [22,23].

However, although the beneficial effects of vitamin D on the prevention or treatment of a variety of oral pathologies have been well-documented, the potential therapeutic role of vitamin D as a supplementary treatment for FCGS following surgical intervention, the primary approach to managing FCGS, has never been reported. Moreover, while the functional activity of vitamin D on the improvement of mucosal lining after FCGS would exert through the VDR-mediated autophagy still remains further explored. With regard to this viewpoint, this study is aimed to examine, first, whether postoperative supplementation with vitamin D would significantly enhance the therapeutic efficacy of surgical intervention for the treatment of FCGS, and therefore successfully improve the quality of life of affected cats. Second, this work also seeks to determine whether autophagic regulation would serve as the underlying mechanism for the enhanced effects of vitamin D activity.

2. Materials and methods

2.1. Experimental groups and surgical procedure

This study utilized a prospective approach, enrolling 18 adult cats (body weight approximately 4.3–5.6 kg) from the Hello Peter Veterinary Hospital (Taichung, Taiwan) that suffered from FCGS and underwent mucogingival replacement surgery (MGRS). Prior to the surgery, complete blood count (CBC), biochemistry and FIV/FeLV tests were performed to ensure that all cats were in a physical condition suitable for general anaesthesia. The surgical procedure was slightly modified from that of Casavecchia et al. and Lewis et al. [24,25]. After intramuscular anesthetizing with Zoletil®50 (tiletamine and zolazepam, 0.02 mL/kg) (Virbac, Kansas City, MO, USA) and Dexdomitor (dexmedetomidine hydrochloride, 0.02 mL/kg) (Zoetis, Parsippany, NJ, USA), ultrasonic tips were first applied to remove the calculus and plaque from the oral cavity, exposing all inflamed areas (as the calculus and plaque could have obscured some of the lesions). Once the extent and location of the lesions were determined, the inflamed oral mucosal tissues were removed through gingivectomy or curettage, and a diode laser (810 nm) was used to achieve haemostasis, sterilization and pain relief at the wound site. The goal of inducing fibrotic scar tissue and collagen coverage over the original wound was to leverage the properties of these tissues, which are less prone to inflammation and therefore more conducive to the healing of the mucosal lining. Additionally, as an attempt to prevent post-operative infections, all cats received oral antibiotics (Doxycycline, 5 mg/kg BID) (Zoetis, Parsippany, NJ, USA). Following surgery, all cats were randomly divided into three groups of six cats in each. The control group did not receive any vitamin D, while the FatVitD group and the WaterVitD group received 200 ng/kg of fat-soluble vitamin D (Vitamin D drops, Panion & BF Biotech Inc., Taipei, Taiwan) and 200 ng/kg of water-soluble vitamin D (Vitamin D drops 100, Panion & BF Biotech Inc., Taipei, Taiwan) via oral administration twice per day for 6 weeks, respectively. During the experimental period, the body weight and the stomatitis disease activity index (SDAI) scores were recorded to evaluate the animal's quality of life at 0, 2, 4 and 6 weeks after surgery. The SDAI scores is assessed based on three major indicators: the cat's quality of life observed by the owner at home (appetite, activity level, grooming behaviour and perceived comfort, scored from 0 to 3), weight changes (scored from 0 to 3) and eight sites of oral inflammation (including maxillary buccal mucosa, mandibular buccal mucosa, maxillary attached gingiva, mandibular attached gingiva, lateral to palatoglossal folds, molar salivary gland, oropharynx and lingual/sublingual area, scored from 0 to 24). In addition to assessing the quality of life, biochemical data were obtained by collecting blood samples every 2 weeks and inflamed oral tissues at the beginning and end of the experimental period (Figure 1). In the care and handling of all experimental animals, the Guide for the Care and Use of Laboratory Animals (1985) as stated in the United States NIH guidelines (NIH publication No. 86–23) were followed. In addition, all surgical procedures were also conducted in accordance with the World Small Animal Veterinary Association Global Dental Guidelines released by the World Small Animal Veterinary Association (WSAVA) in 2020, following the standards of Comprehensive Oral Health Assessment and Treatment (CHOAT) outlined therein.

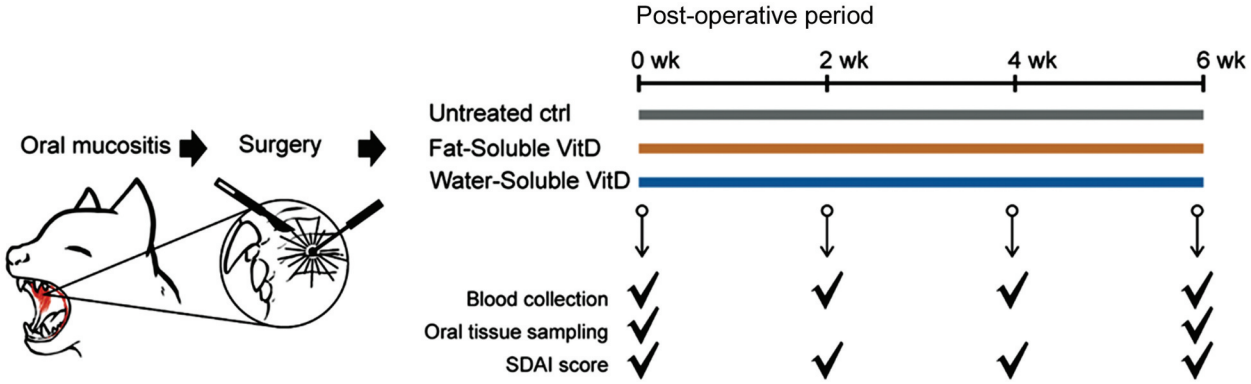

Figure 1. The schematic diagram shows the experimental paradigm of the current study. Cats with oral mucositis (feline chronic gingivostomatitis) treated with mucogingival replacement surgery (MGRS) were administered fat-soluble and water-soluble vitamin D for 6 weeks, respectively. During the experimental period, both blood samples and the stomatitis disease activity index (SDAI) score were collected and recorded at post-operative 0, 2, 4 and 6 weeks. The oral tissue sampling was performed at both the beginning and end of the experiment. Animals that did not receive any post-operative vitamin D supplementation served as untreated controls.

2.2. Determination of serum vitamin D levels

The serum was prepared from the blood samples collected once every 2 weeks during the experiment. After centrifuging at 1000–2000 g for 10 min in a refrigerated centrifuge, the total serum 25-hydroxyvitamin D (25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2) levels was determined using a commercial electro-chemiluminescence immunoassay kit (Elecsys® Vitamin D total II assay, Roche Diagnostics, Mannheim, Germany) and expressed as ng/mL.

2.3. Histopathological evaluation

The oral mucosa tissues that were sampled at the start and end of the experimental period were first fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MA, USA) overnight. After fixation, tissues were blocked in paraffin and serially cut into 5-µm-thick sections. Sections were alternatively mounted on gelatinized slides and processed using regular H&E staining to evaluate possible histopathological changes and further immunohistochemical reactivity.

2.4. Immunohistochemical expression of VDR and LC3B reactivity

To detect VDR and light chain 3B (LC3B) immunoreactivity, sections were first placed in 0.01 M phosphate buffer saline (Sigma-Aldrich, St. Louis, MO, USA) containing 10% methanol (Kanto Chemical Co. Inc., Tokyo, Japan) and 3% hydrogen peroxide (Kanto Chemical Co. Inc., Tokyo, Japan) for 1 h to reduce endogenous peroxidase activity. Following this, sections were incubated in blocking medium containing 0.1% Triton X-100, 3% normal goat serum and 2% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO, USA) for 1 h to block non-specific binding. Sections were then incubated with primary antibody against VDR (1:500) (Abcam ab3508, Cambridge, UK) and LC3B (1:50) (Abcam ab239416, Cambridge, UK) in blocking medium at 4°C for 48 h. Lastly, sections were incubated with a biotinylated secondary antibody (1:200) (Vector Laboratories, Burlingame, CA, USA) at room temperature for 2 h, and assessed using a standard avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) procedure with diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as a substrate of peroxidase.

2.5. Computerized quantitative image analysis

The general approach for the quantitative image analysis was similar to our previous studies [26,27]. A computer-based image analysis system (MGDS) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) was used to quantify the staining intensity. A digital camera mounted on the ZEISS microscope (Axioplane 2, Carl Zeiss MicroImaging GmbH, Hamburg, Germany) imaged sections at 50 × bright field magnification, which were displayed on a high-resolution monitor. The mucosal cells reacted for VDR and LC3B were densitometrically measured, and all densitometric readings from each cell were then combined and averaged to obtain the total optical density (TOD). The background staining (BOD) of each section was measured by averaging five random rectangles within the blood vessels. True OD for each section was then expressed by subtracting BOD from TOD, so that each measurement was made in an unbiased way to correct for background. The staining intensities of VDR and LC3B among different experimental groups were then compared using standardized true OD in which higher value of true OD indicate greater immunoreactivity. All images were captured on the same day by the same experimenter to maintain uniform settings as per the beginning of the experiment.

2.6. Statistical analysis

The data obtained from the SDAI scores across different experimental groups were analysed using the Kruskal–Wallis test. All quantitative data acquired from the biochemical and computerized image analysis were first subjected to Kolmogorov–Smirnov test for analysing the pattern of normality. Those qualified were subsequently processed for a one-way ANOVA followed by a Bonferroni *post hoc* test. Statistical difference was considered significant if $p < 0.05$.

3. Results

The SDAI score was used to assess the life quality of cats suffering from severe oral mucositis and were treated with surgery. The data revealed that in the control group (without post-operative vitamin D supplementation), the mean SDAI scores at post-operative 0, 2, 4 and 6 weeks were 11.90, 8.81, 9.92 and 10.65, respectively (Figure 2A). However, in cats received vitamin D supplementation after surgery, the mean SDAI scores at post-operative 6 week were noticeably increased to 12.29 in FatVit D group and 13.38 in WaterVit D group, respectively (Figure 2A). Although the values among these experimental groups did not reach statistical significance, the results indicate that post-operative supplementation with either fat-soluble or water-soluble vitamin D progressively improves the quality of life for cats suffering from FCGS and treated with surgery.

The serum level of vitamin D was assessed at post-operative 0, 2, 4 and 6 weeks following surgical treatment targeting severe oral mucositis. The data revealed that the average concentration of serum vitamin D was similar in both control (without post-operative supplementation with vitamin D) and WaterVitD (receiving post-operative supplementation with water-soluble vitamin D) groups at all post-operative time points (Figure 2B). Besides, in cats suffering from severe oral mucositis and treated with surgery, the serum level of vitamin D in the FatVitD group (receiving post-operative supplementation with fat-soluble vitamin D) remained unchanged up to post-operative week 4, compared with the control and WaterVitD groups (Figure 2B). However, it is worth noting that after post-operative supplementation with fat-soluble vitamin D for 6 weeks, the serum level of vitamin D significantly increased to 46.7 ng/mL, compared to 38.0 ng/mL in the WaterVitD group and 37.3 ng/mL in the control group (Figure 2B).

The morphological profiles of tissue inflammation were determined by histopathological examination of the samples collected immediately after the surgical intervention and at 6 weeks following the surgery. The results indicated that in samples collected immediately after the surgical intervention, numerous

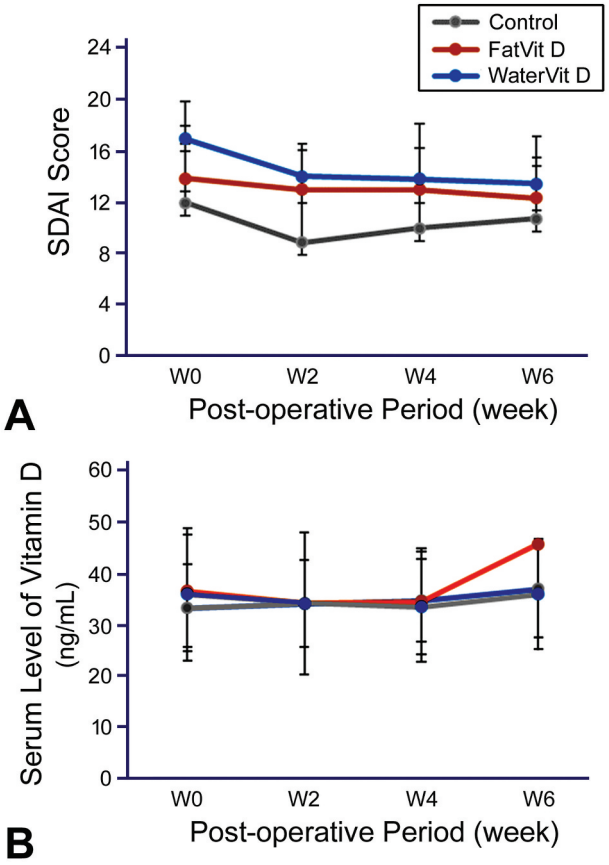

Figure 2. The line graphs show the changing pattern of the stomatitis disease activity index (SDAI) score (A) and the serum level of vitamin D (B) measured at 0, 2, 4 and 6 weeks post-operatively. Note that a noticeable improvement in quality of life (as indicated by the reduction of the SDAI score from post-operative week 0 to post-operative week 6) was observed in the animals that received both water-soluble and fat-soluble vitamin D (A). In addition, the serum level of vitamin D in the control group remained nearly unchanged throughout the entire experimental period (B). However, in animals that received post-operative supplementation with fat-soluble vitamin D, the serum level of vitamin D was significantly up-regulated at 6 weeks post-operatively compared to that in animals receiving water-soluble vitamin D (B). The discrepancy between these two groups may result from the different absorptive and metabolic pathways associated with fat-soluble and water-soluble vitamin D.

inflammatory cells, such as neutrophils, lymphocytes, monocytes and macrophages, were highly infiltrated around the lesion tissues of all experimental animals (Figure 3). In addition, the pathological feature of inflammatory cell infiltration persisted up to post-operative week 6 in animals that did not receive any vitamin treatment. However, in cats that were subjected to surgical treatment and received post-operative vitamin supplementation, the extent of inflammatory cell infiltration was significantly reduced (Figure 3). Although the reduction in inflammatory cell infiltration did not exhibit a significant difference between FatVitD and WaterVitD groups, these data still clearly demonstrated that post-operative supplementation with vitamin D could

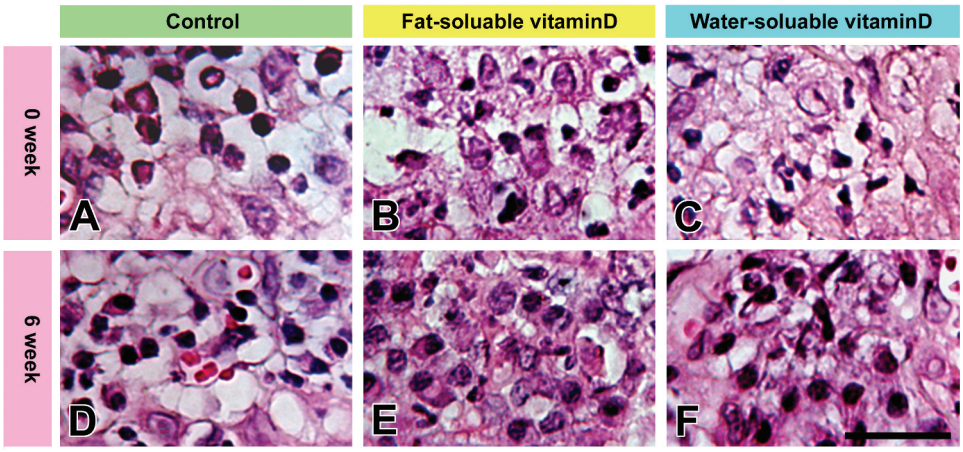

Figure 3. Photomicrographs show the morphological features of the oral mucosa collected from the animals suffering from severe oral mucositis and subjected to surgical intervention, followed by supplementation with non (A, D), fat-soluble (B, E) or water-soluble (C, F) vitamin D at post-operative weeks 0 (A–C) and 6 (D–F). Note that significant inflammation with inflammatory cell infiltration was detected in all experimental groups at post-operative week 0 (A–C). However, in animals that received post-operative supplementation with either fat-soluble or water-soluble vitamin D, the inflammatory state was considerably improved at post-operative week 6 (E, F). Scale bar = 100 µm.

effectively depress the histopathological features of inflammatory reaction in the lesion tissues induced by feline chronic gingivostomatitis.

As an attempt to evaluate the potential benefits of vitamin D in promoting the VDR-mediated cellular processes of autophagy, both VDR and LC3B immunoreactivities were applied to determine the upstream regulator and the downstream essential indicator of autophagic activity, respectively. The present results indicated that in cats suffering from severe oral mucositis and treated with surgery, only a small portion of epithelial cells were positively stained for VDR and LC3B immunoreactivity at 6 weeks following surgery (Figures 4A,D, 5A,D). However, in cats suffering from severe oral mucositis and undergoing surgery, post-operative supplementation with vitamin D remarkably increased both VDR and LC3B immuno-expression at 6 weeks following surgery (Figures 4B,C,E,F, 5B,C,E,F). Quantitative data corresponded well with the immunohistochemical findings, with the true OD of the VDR and LC3B immuno-staining measured at post-operative 6 weeks being significantly higher in animals receiving post-operative supplementation with vitamin D compared to those without vitamin D administration post-operatively (Figure 6). In addition, the up-regulation of VDR and LC3B immuno-expression was more intense in animals supplied with fat-soluble vitamin D than in those receiving water-soluble vitamin D (Figure 6).

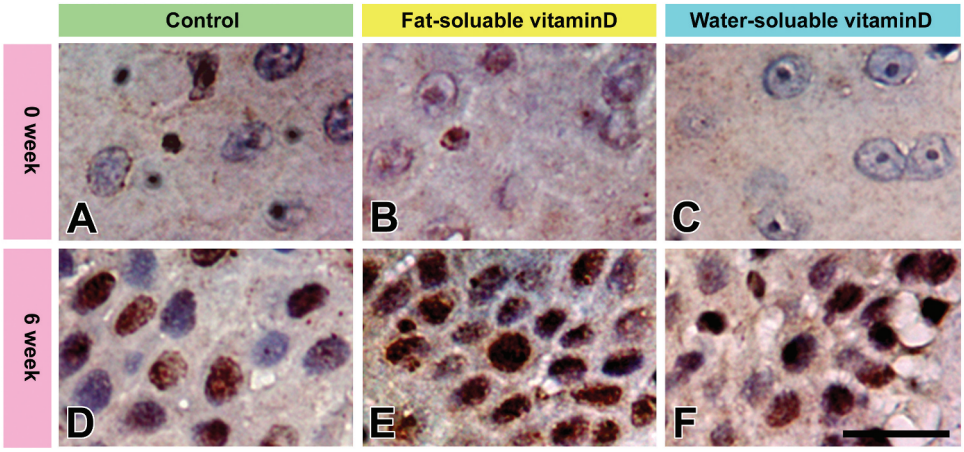

Figure 4. Photomicrographs show the vitamin D receptor (VDR) immuno-expression in animals suffering from severe oral mucositis and subjected to surgical intervention, followed by supplementation with non (A, D), fat-soluble (B, E) or water-soluble (C, F) vitamin D at post-operative weeks 0 (A–C) and 6 (D–F). Note that there was nearly no or very weak VDR staining in all experimental groups at post-operative week 0 (A–C). However, the immunoreactivity of VDR was significantly increased at post-operative week 6 following supplementation with both fat-soluble and water-soluble vitamin D (E, F). Scale bar = 100 µm.

4. Discussion

This study is the first to provide functional anatomical evidence suggesting that post-operative supplementation with vitamin D may be a valuable strategy to enhance the success of surgical interventions dedicated to the treatment of FCGS. Such supplementation offers several benefits for cats with FCGS undergoing surgery. It significantly increases serum level of vitamin D (Figure 2), effectively reduces inflammatory cell infiltration (Figure 3), and remarkably enhances VDR-mediated autophagy (Figures 4, 5, 6). These effects collectively contribute to a substantial improvement in the quality of life of affected cats (Figure 2). In addition, the beneficial effect of vitamin D on promoting autophagy is more evident in the animals given fat-soluble form of vitamin D than that receiving water-soluble one (Figures 4, 5). It is well indicated that vitamin D is an essential organic compound best known for its functional roles in calcium homoeostasis and bone remodelling [11]. Over the past few decades, the beneficial effects of vitamin D on preserving the oral health have garnered significant attention, particularly due to its potent anti-inflammatory activity [12,28–31]. Previous studies have indicated that supplementation with vitamin D could effectively reduce the incidence of periodontitis, dental caries, oral squamous cell carcinoma and radiation-induced oral mucositis [13–15,17,32,33]. Pharmacological reports also demonstrated that vitamin D deficiency is positively associated with the increased risk of chronic periodontitis, dentin/enamel defects, gingival inflammation and osteonecrosis of the jaw [34–37]. Through significantly elevating serum vitamin D levels, the synthesis of proteins essential for enhancing the oral mucosal stability would greatly increase, which subsequently leading to successful improvements in overall health of the oral cavity [30,38]. This is the case that in our present study, we also detected an increased serum level of vitamin D in FatVitD group after 6 weeks of post-operative supplementation (Figure 2). The increment of the serum level of vitamin D corresponded well with the reduced inflammatory cell infiltration (Figure 3) and better life quality (as measured by decreased SDAI scores, Figure 2). However, it is interesting to note that the serum level of vitamin D did not exhibit significant difference between WaterVitD group and that of the control ones (Figure 2). Although the exact cause of this phenomenon is not fully understood, differences in the absorption and metabolism of fat-soluble and water-soluble vitamins are suggested to account for this discrepancy. It has been reported that fat-soluble vitamins can only be absorbed by the organisms after being encapsulated in fat globules [39]. Excess fat-soluble vitamins are stored in the liver or adipose tissue and released as needed [39]. Our present findings thus align well with this perspective in which the serum level of vitamin D in FatVitD group began to increase from post-operative 4 weeks, presumably caused by the gradual release of stored fat-soluble vitamin D in the body (Figure 2). On the other hand, water-soluble vitamins can be directly dissolved in water, making them easily excreted in urine and less likely to accumulate at high concentrations in the bloodstream [40]. As the beneficial effects of vitamin D on local tissues depend on maintaining adequate concentrations, future studies should investigate whether extending the post-operative supplementation period could enhance its effectiveness in mitigating severe oral mucosal injury.

In addition to exert, the anti-inflammatory function, vitamin D also plays an essential role in regulating autophagy. A variety of biochemical studies have demonstrated that vitamin D could activate autophagy through both genomic and non-genomic signalling pathways [21]. In the genomic action,

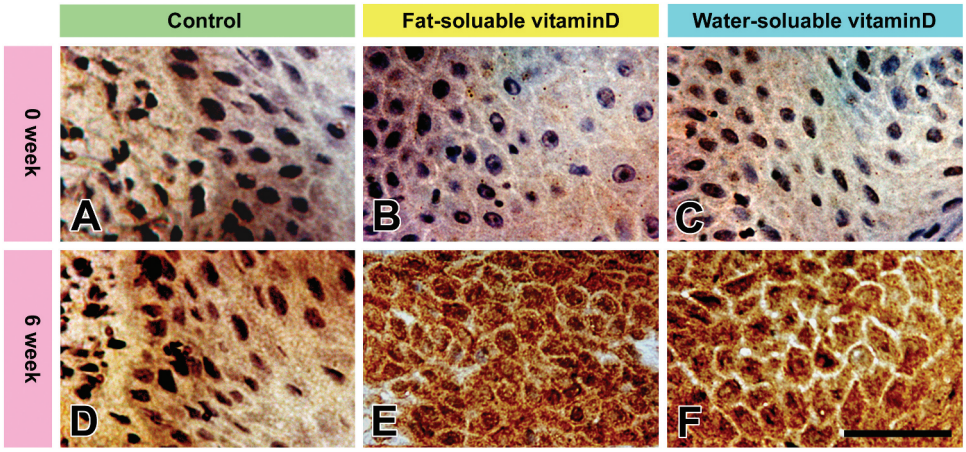

Figure 5. Photomicrograph show the light chain 3B (LC3B) immuno-expression in animals suffering from severe oral mucositis and subjected to surgical intervention, followed by supplementation with non (A, D), fat-soluble (B, E) or water-soluble (C, F) vitamin D at post-operative weeks 0 (A–C) and 6 (D–F). Note that the immunoreactivity of LC3B in animals that received post-operative supplementation with both fat-soluble (E) and water-soluble (F) vitamin D was remarkably increased at post-operative week 6 compared to that of the control group (D). Scale bar = 100 μm.

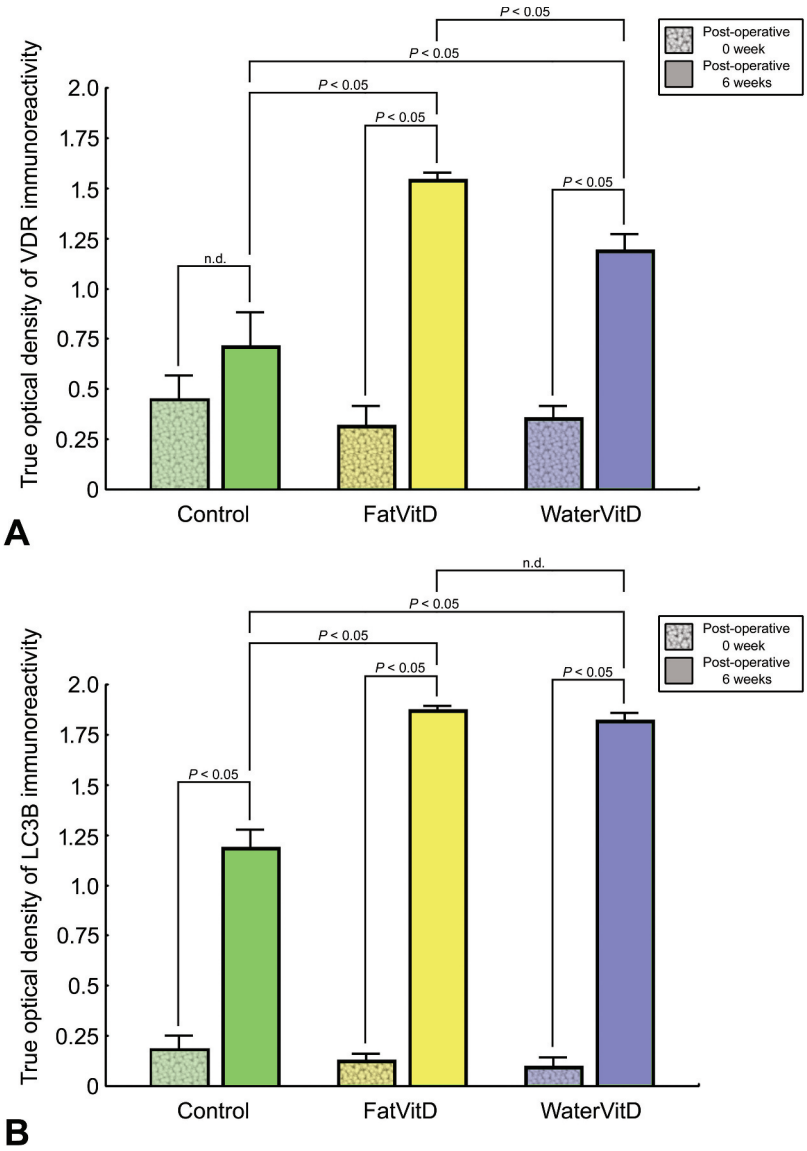

Figure 6. Histograms illustrate the staining intensity (as measured by the true optical density) of the vitamin D receptor (VDR) (A) and light chain 3B (LC3B) immuno-expression (B) in animals suffering from severe oral mucositis and undergoing surgical intervention, with or without post-operative supplementation with vitamin D. Note that the staining intensity of VDR was significantly increased at post-operative week 6 in animals that received post-operative supplementation with both fat-soluble and water-soluble vitamin D (A). The expression pattern of LC3B correlated well with the VDR staining, with post-operative supplementation with both forms of vitamin D effectively enhancing the staining intensity of LC3B at 6 weeks post-operatively (B). It is noteworthy that the LC3B staining was also significantly increased at post-operative week 6 in control animals that did not receive any vitamin D supplementation (B). This result may be linked to the cytoskeletal functions of LC3B involved in the process of wound repair, beyond its role as a biomarker of autophagy.

vitamin D could bind to VDR to up-regulate autophagy-related genes, including LC3B, ATG5 and BECN1, to control inflammation and enhance host immunity [21,41]. For the non-genomic mechanism, vitamin D would act via the membrane-binding receptors to regulate various signalling pathways [such as phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)] to trigger autophagy and alleviate inflammatory reactivity [21,42]. In a good agreement with these studies, our present study also detected an enhanced VDR expression at 6 weeks following post-operative supplementation with both forms of vitamin D (Figures 4, 6). Augmentation of VDR further coincided well with the increased expression of LC3B at six-week post-operatively (Figures 5, 6). These data clearly demonstrate that post-operative supplementation with vitamin D certainly activates VDR-mediated autophagy, mainly through the LC3B pathway. However, it is noteworthy that the LC3B immuno-expression was also significantly increased in the control group that did not receive any administration of vitamin D (Figure 6). Since LC3B also functions as a microtubule-associated protein (MAP) and regulates the mRNA expression of fibronectin [43,44], the increased expression of LC3B in the control

group may not be associated with autophagy, but rather result from fibronectin-mediated cytoskeleton reconstruction and subsequent wound repair following surgical intervention for the treatment of severe oral mucosal injury. Nevertheless, while this study clearly demonstrates the beneficial effects of vitamin D supplementation after mucogingival surgery in improving life quality and enhancing autophagy in cats with FCGS, limitations related to the sample size and other mechanisms of vitamin D in autophagy regulation should not be overlooked. Recruiting more animals for this study and investigating the functional roles of the non-genomic mechanisms of vitamin D (such as PI3K/AKT pathway) in triggering autophagy and anti-inflammatory responses will provide deeper insights into its therapeutic potential and lay the foundation for future studies to refine treatment strategies for cats with severe oral mucosal injury.

5. Conclusion

In this study, we provided the first functional anatomical evidence that post-operative supplementation with vitamin D (200 ng/kg twice daily for 6 weeks) can effectively reduce the extent of inflammatory cell infiltration, promote VDR-mediated autophagy and improve the quality of life in cats suffering from FCGS. As low doses of vitamin D are generally considered beneficial and safe for most cats, these findings offer a valuable and practical strategy for clinicians to enhance the success rate of surgical intervention aimed at counteracting lesions caused by severe oral mucosal injury.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, HMC, upon reasonable request.

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