Homeopathic *Viscum Album* at Potencies D3 and 200CH Presents Cytokine Modulatory Effect Produced by *in vitro* Culture of Mesenchymal Stem Cells

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**Citation:** Valle ACV, Brunel HSS, Malard PF, Villarroel CL, Andrade RV (2022) Homeopathic *Viscum Album* at Potencies D3 and 200CH Presents Cytokine Modulatory Effect Produced by *in vitro* Culture of Mesenchymal Stem Cells. Curr Res Cmpl Alt Med 6: 155. DOI: 10.29011/2577-2201.100055

**Received Date:** 12 May 2022; **Accepted Date:** 23 May 2022; **Published Date:** 27 May 2022

**Abstract**

*Viscum Album* (VA) has been widely used as adjuvant therapy in cancer. Its anticancer activity has been attributed to a combination of cytotoxic, immunomodulatory, and anti-inflammatory properties, induction of apoptosis, and inhibition of angiogenesis. This study aimed to evaluate the *in vitro* immunologic effects of homeopathic VA on mesenchymal stem cells. Two concentrations of VA in the potencies D3 and 200CH were used: 20 and 30 µL/mL. The levels of the pro-inflammatory cytokines TNF-α, IFN-γ, IL-6, and IL-1β produced by the cells were measured after a 48h-incubation period with VA. The concentrations of VA D3 and VA200CH used did not induce changes in TNF-α and IFN-γ responses but decreased the values of IL-6 and IL-1β. This result demonstrates that VA D3 and VA200CH may play an important role as anti-inflammatory and immunomodulatory medicines.

**Introduction**

*Viscum Album* (VA) is a hemiparasitic plant of the Loranthaceae family used to treat many diseases in traditional and folk medicines. Anticancer, immunomodulatory, antidiabetic, neuropharmacological, antibacterial, hepatoprotective, cardiac, and antifungal properties comprise the most studied properties of VA extracts [1].

Extracts produced from VA are used for treating cancer within concepts of complementary medicine, especially anthroposophical medicine. Several studies have reported the modulatory effect on the natural immune system of VA extracts. These findings may explain the therapeutic effects observed in some patients with cancer, such as prolonged overall survival, improved quality of life, and reduction of adverse events associated with conventional anticancer treatments [2,3]. Studies have also found that homeopathic VA can reduce the viability of cancer cells *in vitro* [4,5].

According to Gardin [45], VA can enhance the immune response and restore cellular and humoral immunosuppressed systems. Its bi-directional activity in cancer treatment has been widely documented. Initially, it affects the quality of life of cancer patients...
patients by showing a supportive effect on cancer-related fatigue, insomnia, exhaustion, nausea, vomiting, appetite, depression, anxiety, pain, physical functioning, and side effects of traditional treatments [6,7]. However, the molecular and cellular mechanisms behind the beneficial effects of VA encompass the antitumor activity by cytotoxic properties, induction of apoptosis [8,9], and inhibition of angiogenesis [10]. Findings indicate that adjuvant treatment of cancer patients with VA can be associated with better survival [11,12].

Its extracts contain various substances, such as lectins, viscotoxins, alkaloids, lignans, sterols, fatty acids, flavonoids, terpenoids, phenolic acids, and phenylpropanoids. Lectins are the major biologically active components of the VA extracts. They are known to induce apoptosis in lymphocytes and tumor cells, and at toxic concentrations, they can also stimulate the release of cytokines [13].

Cytokines are small and soluble proteins or glycoproteins that mediate cell-to-cell communication. They are secreted by various immune and nonimmune cells and are responsible for growth, differentiation, and inflammatory or anti-inflammatory signals to diverse cell types [14,15]. Cytokine-mediated effects dominate the fields of inflammation, immunology, atherosclerosis, and cancer [16]. Several cytokines limit tumor cell growth by a direct antiproliferative or pro-apoptotic activity or indirectly by stimulating the cytotoxic activity of immune cells against tumor cells [14].

Cytokines like IFN-γ or TNF play a critical role in creating an immunogenic microenvironment and, consequently, fighting against metastatic cancer. IFN-γ is produced predominantly by NK cells and natural killer T (NKT) cells as an innate immune response. TNF-α is a pleiotropic cytokine involved in multiple homeostatic and pathological mechanisms. It is mainly produced by macrophages, T lymphocytes, and NK cells. It is a multifunctional cytokine involved in apoptosis, cell survival, inflammation, and immunity [17].

IL-6 is also a pleiotropic cytokine, and the increase in its expression has been linked to the pathogenesis of several disorders such as chronic inflammatory diseases, autoimmune diseases, and tumor development. This feature could be explained by its influence on the cellular metabolism and several immune and physiological processes, such as antigen-specific immune responses, inflammation, hematopoiesis, apoptosis, and differentiation [18,19,20]. Excessive IL-1β production contributes to autoimmune [21] and causes autoimmune diseases [22]. In addition, in the case of chronic inflammation, sustained IL-1β may promote both tumor induction and, later on, also tumor propagation by different mechanisms [23]. This cytokine has also contributed to the severity of inflammatory diseases [24,25].

Clinical trials have increasingly assessed the safety and efficacy of cytokine-based drugs in recent years due to a renewed interest in the antitumor properties of cytokines. Consequently, cytokines have been studied as single agents and combined with other immunomodulatory drugs [14].

Therefore, cytokines are being measured and monitored more routinely in clinical laboratories due to their enhanced use as therapeutics, targets for modulation of undesirable inflammation, biomarkers of inflammation, and diagnostic markers [15].

The objective of this study was to assess the in vitro immunologic effects of homeopathic VA on mesenchymal stem cells by dosing IL-6, IL-1β, TNF-α, and IFN-γ.

Methods

Cell culture

Mesenchymal stem cells (MSC) derived from adipose tissue of two donors were obtained from a commercial cell bank. Cells were cultured with Dulbecco’s Modified Eagle Medium (DMEM) added with 10% fetal bovine serum and 0.02% amikacin (all from the Sigma-Aldrich® brand) following the Data Sheet methodology.

Preparation of VAD3 and VA200CH

The Mother Tincture was the starting point for preparing the tested substance (VAD3 and VA200CH). As described in the Brazilian Homeopathic Pharmacopoeia, the Hahnemannian Decimal Method was used. Using a sterile isotonic solution, 1 part of the active ingredient was mixed with 9 parts of the inert ingredient and succussed 100 times, yielding VAD1 (1×10−1). Then, 1 part of VAD1 was used with 9 parts of the inert ingredient and succussed 100 times, yielding VAD2 (1×10−2). The successive dilution continued till VAD3 and VA200CH were obtained. These products were then bottled in 1.1 mL ampoules.

Cytokines analyses

Cells were plated in 6-well plates (1x10⁵ cells per well) with culture medium (DMEM with 10% FBS) and incubated at 37°C and 5% CO₂ for 24 hours. After this period, the treatment of cells was initiated with the culture medium containing different concentrations of homeopathic VA (VAD3 and VA200CH) at 20 and 30 µL/mL of culture medium, followed by incubation for 48 hours in the conditions previously described. Control wells were maintained containing cells and culture medium with no addition of VA. After 48 hours of culture, the medium was collected and frozen to further quantify cytokines IL-6, IL-1β, TNF-α, and IFN-γ. Analyses were performed in triplicate wells, and the dosage was calculated automatically based on the kit standards. The cytokines dosages (pg/mL) were performed by the sandwich ELISA method with commercial kits (Prepotech mini-ABTS), following the datasheet provided by the manufacturer. The standards and samples were blank.
were distributed in 96-well plates. Reading was performed in a spectrophotometer at a 405 nm wavelength and correction at 650 nm.

**Statistical Analysis**

Data are presented as means and standard deviations. The one-way analysis of variance was used to compare each group with the control. Results were considered significant at $P < 0.05$. All statistical analyses were performed GraphPad Prism version 7.0.4 for Windows, GraphPad Software, San Diego, California USA, “www.graphpad.com”.

**Results**

In our study, mesenchymal stem cells from two donors were cultured in the presence of different concentrations of homeopathic VA. The cytokine dosages in the supernatant were measured, and the mean and standard deviation was calculated as presented in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/mL)</th>
<th>IFN-γ (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.930 ± 11.802</td>
<td>47.488 ± 10.428</td>
<td>526.158 ± 62.957</td>
<td>69.611 ± 3.954</td>
</tr>
<tr>
<td>VAD3 20</td>
<td>18.989 ± 2.137</td>
<td>28.105 ± 4.038</td>
<td>404.49 ± 45102</td>
<td>52.863 ± 5.696</td>
</tr>
<tr>
<td>VAD3 30</td>
<td>21.131 ± 3.660</td>
<td>27.262 ± 1.891</td>
<td>406.773 ± 39.004</td>
<td>37.635 ± 6.382</td>
</tr>
<tr>
<td>VA200CH 20</td>
<td>21.322 ± 4.546</td>
<td>23.009 ± 0.940</td>
<td>396.686 ± 77.523</td>
<td>47.315 ± 8.125</td>
</tr>
<tr>
<td>VA 200CH 30</td>
<td>23.543 ± 6.067</td>
<td>31.644 ± 2.292</td>
<td>421.232 ± 65.278</td>
<td>50.669 ± 5.036</td>
</tr>
</tbody>
</table>

Table 1: Mean and standard deviation of cytokines dosed in the study

The present study results demonstrate that the VA concentrations of 20 and 30 μL/mL were not sufficient to induce changes in TNF-α and IFN-γ responses in mesenchymal stem cells. However, it is possible to observe a decreasing trend of these cytokines with these treatments (Figure 1).

**Figure 1**: TNF-α and IFN-γ responses to VAD3 and VA200CH. Data presented by mean and standard deviations.

The pro-inflammatory cytokines IL-6 and IL-1β decreased ($P<0.05$) in the supernatant when VA was added to the culture medium (Figure 2), meaning that the cells produced less IL-6 and IL-1β.
Discussion

Our study demonstrates that different concentrations of VAD3 and VA200CH (20 and 30 μL/mL) were not sufficient to induce significant changes in TNF-α and IFN-γ but decreased the pro-inflammatory cytokines IL-6 and IL-1β produced by mesenchymal stem cells.

Cytokines, including interleukins, interferons, and tumor necrosis factors, have various pro- and anti-inflammatory effects in the body through several biochemical pathways and interactions [26]. TNF-α is a pro-inflammatory cytokine critical for cell communication during host defense, inflammation, and organogenesis. TNF-α plays a central role at the beginning of the inflammatory reactions of the innate immune system. This cytokine can cause chronic inflammation if not regulated and septic shock when high acute concentrations are generated. In a study with mice in which TNF-α mRNA stability was increased, they developed arthritis, cachexia, and inflammatory bowel disease [27]. These mouse experiments relate directly to recent studies in chronic human diseases since deregulated local production of TNF-α is characteristic of diseases such as rheumatoid arthritis [28] and Crohn’s disease [29]. Treatment with TNF-α antagonists is effective in most patients. In our study, the non-alteration of TNF-α observed in cells treated with VAD3 and VA200CH suggests that these medicines may bring benefits with the potential to regulate the immune system, avoiding the high release of TNF-α. Many chronic inflammatory diseases are associated with an increased risk of cancer, as these cell mutations arise at sites of chronic inflammation and chemical mediators of inflammation are found in many cancers [30, 31] and one of the major chemical mediators implicated in inflammation-associated cancers is TNF-α. There is substantial evidence that TNF-α is involved in the promotion and progression of cancers in humans since, when produced in the tumor microenvironment, TNF-α can act as an endogenous tumor promoter [32]. Orosz [33] showed increased lung metastases of an experimental fibrosarcoma after pre-treatment of animals with TNF-α. Alternatively, lung metastases may be reduced by adding an anti-TNF-α antibody, neutralizing endogenous TNF-α levels. Therefore, medicines that regulate the release of this factor in vitro, such as VAD3 and VA200CH, may be important as aids in treating cancer.

Another cytokine studied in this study, IFN-γ, promotes both protective immune responses and immune-pathological processes. In response to inflammatory mediators, including IFN-γ, tumor cells initiate an immune regulation program [34,35]. Under the influence of chronic inflammation associated with cancer, IFN-γ can thus induce the expression of immune regulatory molecules in tumor cells. Immunogenic cancers such as melanoma, prostate cancer, breast cancer, leukemia, and HPV-infected high-grade cervical intraepithelial neoplasia can stimulate IFN-γ production upon involvement with immune cells [36]. In addition, immunogenic cell death is widely observed in solid tumors and induces the STING molecule (IFN/TMEM173 gene stimulator) that stimulates interferon production, including IFN-γ [37,38]. IFN-γ-mediated increase in various molecules such as CD38 in chronic lymphocytic leukemia and CD74 in melanoma can directly contribute to tumor cell proliferation and survival [39,40]. A medicine that regulates the production of IFN-γ, not allowing the production of high levels of this cytokine, may be important in treating cancers. By not stimulating the in vitro production of this molecule, VAD3 and VA200CH may become essential tools to be better studied in the complementary treatment against tumors.

The decrease of pro-inflammatory cytokines in tumor environments may be one of the critical mechanisms for cancer control. IL-6 is a pleiotropic cytokine and can influence several
immune and physiological processes, such as inflammation, antigen-specific immune responses, hematopoiesis, apoptosis, differentiation, and cellular metabolism [18,19,20]. Increased IL-6 expression has been linked to the pathogenesis of several disorders, such as chronic inflammatory diseases, autoimmune diseases, and tumor development [41]. Also, the IL-6/IL-6R axis contributes to the progression of several diseases, and inhibition of this axis is highly effective against diseases such as rheumatoid arthritis (RA), Castleman disease, and cytokine release syndrome [42]. The low in vitro production of this cytokine by mesenchymal stem cells stimulated by the homeopathic medicines VAD3 and VA200CH may be an important factor since this medicine is used in the integrative treatment of some types of cancers [43, 44] and due to its role as an immune system stimulator and ability to reestablish immune-suppressed cellular and humoral systems [45].

In conjunction with the low production of IL-6, our study also demonstrated the low IL-1β production, another cytokine related to inflammation and cancer cases. Excessive IL-1β production contributes to autoimmune [21] and causes autoinflammatory diseases [22]. Sustained IL-1β caused by chronic inflammation may promote both tumor induction, tumor propagation [23] and contribute to the severity of inflammatory diseases [24,25]. For example, elevated IL-1 signaling was demonstrated to cause neuronal cell death [46]. Of note, high IL-1β production has been observed in patients who have epilepsy [47], stroke [48], Alzheimer’s disease [49], and other neurological disorders [50].

There is a relationship between the cytokines presented in this study: the innate pro-inflammatory cytokines, including IL-1, TNF-α, and IL-6, are crucial to resolving acute inflammations. However, high levels of innate cytokines, as apparent in chronic inflammation, may promote tumor development by driving sustained NF-κB activation [51] and mitogen-activated protein kinase (MAPK) activity [52]. The driving mechanism is the high-level production of IL-1β by plasma cells which induces IL-6 in stromal cells [53]. IL-6, in turn, promotes the development of malignant plasma cells. Increased levels of IL-1β in body fluids are correlated in experimental tumor models and cancer patients with bad prognosis, carcinogenesis, and experimental tumor models and cancer patients with bad prognosis, carcinogenesis, and invasiveness of the tumor [54]. The key mechanisms by which IL-1β promotes tumor development are driving chronic non-resolved inflammation [55], endothelial cell activation [56], and chronic tumor angiogenesis [54] and induction of immunosuppressive cells. Altogether, these mechanisms account for suppression of the adaptive immunity, tumor promotion and metastasis suppression of the adaptive immunity, tumor promotion, and metastasis [55]. In this context, it is vital to downregulate the production of these cytokines to prevent or control cancer diseases, as VAD3 and VA200CH possibly did in our in vitro study.

**Conclusion**

Since TNF-α, IFN-γ, IL-6, and IL-1β have pro-inflammatory effects, the non-alteration of the tumor factor and the interferon proteins favors the role of VAD3 and VA200CH as an anti-inflammatory medicine. Also, the decrease in IL-6 and IL-1β produced by cells treated with VA demonstrates that VAD3 and VA200CH may play an important role as anti-inflammatory and immunomodulatory medicines. Despite the interesting in vitro results observed, more in vivo studies should be performed to record the direct relationship of the effect of these medicines against the various types of cancers.

**References**


