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An Exploration of Homeopathy: The Effect of Triterpenoids on Placental Cell Lines

The placenta is a transient yet highly specialized organ of pregnancy, crucial for supporting the normal growth and development of a fetus in utero. It facilitates the transfer of nutrients and waste products between maternal and fetal circulatory systems. Therefore, studying the placenta is essential to understand whether external stressors on the mother affect the fetus during pregnancy. Deviations in placental development or function can have dramatic effects on the fetus and its survival (Gude et al., 2004).

The placenta is comprised of multiple cell types, each associated with different functions during gestation. Villous stem cell cytotrophoblasts (VSCCs), originating from the outermost layer of the blastocyst, eventually develop into a large portion of the placenta. They are the first cells to differentiate from a fertilized embryo into placenta-designated cells. VSCCs then differentiate into either extravillous trophoblasts (EVTs) or villous cytotrophoblasts (VCT). EVTs give rise to both endovascular trophoblast cells, remodeling uterine arteries, and interstitial trophoblast cells, migrating into the decidua and myometrium for placental attachment. VCTs differentiate into syncytiotrophoblast cells, the primary site of placental transport and perform protective and endocrine functions within the placenta (Gude et al., 2004).

Immortalized/cancerous cell lines are commonly used in research to study cellular mechanisms due to their indefinite lifespan, which facilitates continuous cell culture. While immortal cell lines may offer insights into placental cell development and function in vitro, they are not a complete replacement for primary cells, which may respond differently to stressors (Kaur & Dufour, 2012). In our proposed study, we will perform cell culture using three immortalized cell lines derived from human cells: undifferentiated BeWo and JEG-3 cells (models for VCTs), and HTR8/SVNeo cells (a model for EVTs) (Block and Monahan, 2024).

For thousands of years, Asian countries have utilized plants for their medicinal properties. The consumption of plants containing triterpenoids, a class of compounds with a steroidal backbone, has been associated with preventing and treating various human diseases, including cancer and immunological disorders, as well as enhancing vitality and longevity. Some mushrooms, among other eukaryotes, contain triterpenoids and have been investigated for their antitumor roles in humans (Hsu & Yen, 2014). Hossain et al. (2012) found that Ganoderic acid D (GAD) induced autophagic and apoptotic cell death of metastatic melanoma cells *in vitro*. Additionally, triterpenoids have been shown to disrupt the cellular pathways associated with proliferation. Shi et al. (2018) investigated the effect of Tubeimoside-1 (TBSM1), another triterpenoid, on the VEGF-A/VEGFR-2/ERK pathway in non-small cell lung cancer (NSCLC) cells. They found that TBSM1 inhibited the VEGF-A/VEGFR-2/ERK pathway, in turn repressing the growth and metastasis of NSCLC cells. This pathway is also key in placentation, specifically, VEGF gene expression is essential for angiogenesis to occur throughout the body, but especially during early placentation to remodel spiral arteries (Bacenkova, 2022).

According to the NIH, more than 6 million people in the United States utilize homeopathic remedies for specific health conditions, and over 200 million people use homeopathy worldwide (DeWit & Office of Advocacy, 2020). Despite an increasing trend toward the use of homeopathic remedies and the encouraging data regarding the

positive effects of triterpenoids, particularly Ganoderic acids, there is a lack of research of their potential effects on pregnant women and fetal development. Triterpenoids hold promise in aiding mothers in regulating diverse bodily systems to mitigate pregnancy-related conditions. Successful placental function relies on events during the implantation stage at the maternal-fetal interface. Interference with this process can lead to detrimental health outcomes for either mother or fetus, such as preeclampsia, suggested to result from abnormally shallow placentation (Bisson et al., 2023). Cellular proliferation is crucial for placental implantation, prompting the investigation of commonly used drugs or homeopathic remedies that could interrupt or dysregulate this process. Further research is necessary to determine whether Ganoderic acids have a detrimental impact on placental cell lines. In this study, we replicated past methodology to assess the anti-proliferative effects of GAD on cancerous placental cell lines, to explore whether GAD is a safe homeopathic remedy suitable for consumption during pregnancy.

Specific Aims

Hypothesis: Ganoderic Acid D (GAD) will cause widespread disruption of cellular mechanisms in BeWo, JEG-3 and HTR8/SVNeo cell lines.

Aim 1: Determine if GAD causes a negative impact on cell viability. We explored the impact of GAD on trophoblast viability in immortalized cell lines (undifferentiated BeWo, JEG-3, and HTR8/SVNeo) with two viability assays.

Aim 2: Determine if GAD alters internal cellular mechanisms. We investigated the downstream RNA and protein expression of trophoblast cells (undifferentiated BeWo, JEG-3, and HTR8/SVNeo) to determine which cellular mechanisms might be altered in response to treatment with GAD. We lysed the three trophoblast cell lines after treatment with either PBS or GAD to isolate either RNA or protein. We then ran PCR or western blots accordingly. We explored the impact of GAD on GLUT1, MMP9, and VEGF-A, with GAPDH as a housekeeping gene. GLUT1 is a gene that encodes a glucose transport protein across the placenta, that is essential for glucose homeostasis during pregnancy. MMP9 is a gene that encodes for an enzyme that breaks down the extracellular matrix, which is essential for extravillous trophoblast invasion of the maternal endometrium. Lastly, VEGF-A is an endothelial growth factor that has angiogenic properties. It is also essential for trophoblast proliferation and migration. To explore the protein content of our cells treated with GAD, we utilized a western blot technique to visualize cytochrome C expression, which serves as a marker for apoptosis.

Experimental Design

Cell Culture. BeWo, JEG-3, and HTR8-SVNeo immortalized cell lines were provided by the lab, thawed from cryopreservation, and plated as needed. These immortalized trophoblast cell lines were cultured in DMEM/F12, DMEM, and RPMI media respectively, with 10% FBS and 1% penicillin-streptomycin.

RNA Extraction and PCR. RNA was extracted and assessed for quantity and quality using the Nanodrop. Samples failing to meet specified criteria were excluded from downstream analyses. PCR was employed to assess the expression of genes (VEGFA, MMP9, GLUT1, GAPDH) following treatment with GAD. VEGFA and MMP9 are linked to placental vasculogenesis and angiogenesis, GLUT1 is crucial for glucose transport across the placenta, and GAPDH serves as a housekeeping gene/loading control, enabling data normalization by comparing the expression of other genes to GAPDH.

BCA Protein Assay and Western Blot. BCA protein assays determined protein concentrations in samples by comparing them to a standard curve. The curve, prepared using the Pierce BCA Protein Assay Kit, was analyzed with Gen5 software at 562 nm absorbance. Unknown sample concentrations were calculated using the linear equation (y=mx+b) derived from the standard curve. Western Blot assessed the presence of specific proteins after Ganoderic Acid D treatment, including AMPKalpha (phosphorylated and non-phosphorylated forms), cytochrome C, cytokeratin 7, and α-tubulin. AMPKα regulates cell growth, cytochrome C indicates apoptosis-induced mitochondrial damage, cytokeratin 7 marks trophoblasts, and α-tubulin, a housekeeping protein, allows for normalization ofdata..

Cell Staining (ICC). To stain the cells, cell lines were plated onto coverslips in 6-well plates. Cells were fixed with PFA and made permeable with Triton-X. Cells are incubated overnight at 4ºC with primary antibody (Cytokeratin 7, a trophoblast cell marker). The cells are then stained with the appropriate secondary antibody. Cells were then mounted with DAPI-positive mounting media and imaged utilizing the confocal microscope.

Viability Assays. viability analysis was conducted using the CellTiter Glo 2.0 Assay, confirming viable cell counts post-treatment with PBS or Ganoderic Acid D. This assay uses luciferase-catalyzed mono-oxygenation of luciferin, resulting in a luminescent signal. The luminescence directly correlates with ATP levels and the quantity of cells in the culture. Additionally, the RealTime-Glo MT Cell Viability Assay monitored cellular luminescence over time after treatment with PBS or GAD. By introducing NanoLuc luciferase and MT Cell Viability Substrate, the assay measured the reduction of the substrate by metabolically active (viable) cells, allowing for a continuous read format. NanoLuc luciferase utilizes this substrate to generate a luminescent signal, measured by Gen5 software. This approach allowed for real-time tracking of cell viability, essential for discerning the onset of toxicity induced by Ganoderic Acid D.

Results

No Dose-Dependent Effect of GAD on the Viability of BeWo, HTR8/SVNeo, or JEG-3 Cells

In assessing BeWo cell viability through the MT viability assay, luminescence was analyzed as a function of growth across different cell concentrations per well (3000, 6000, and 9000) without GAD treatment (Figure 1A). Notably, a relatively linear trend was observed at 3000 cells/well, whereas the other concentrations displayed a non-linear pattern. 67 hours post-GAD treatment, there was a marked increase in cell death which was evident in comparison to the 44-hour time point as seen by the change to a negative slope. This result suggests that the assay is only reliable up to the 44-hour time point (indicated by the green arrow in Figure 1A). With the understanding that 3000 cells/well yielded the most linear trend in cell growth and recognizing the assay's diminished accuracy beyond the 44-hour mark, going forward we restrict our data analysis to instances where 3000 cells/well are utilized up to the 44-hour time point. To explore the impact of GAD on BeWo cell viability and identify the most influential dosage, we examined cell viability across 8 doses (0 uM, 5 uM, 10 uM, 20 uM, 40 uM, 80 uM, 60 uM, and 100 uM). While we anticipated a dose-dependent curve with 0uM having the greatest cell viability and 100uM having the least, we did not observe this trend. Rather, no discernable dose-dependent trend emerged from our results (Figure 1B). These results indicate a lack of observable effects of GAD on Unsyncitialized BeWo cell viability.

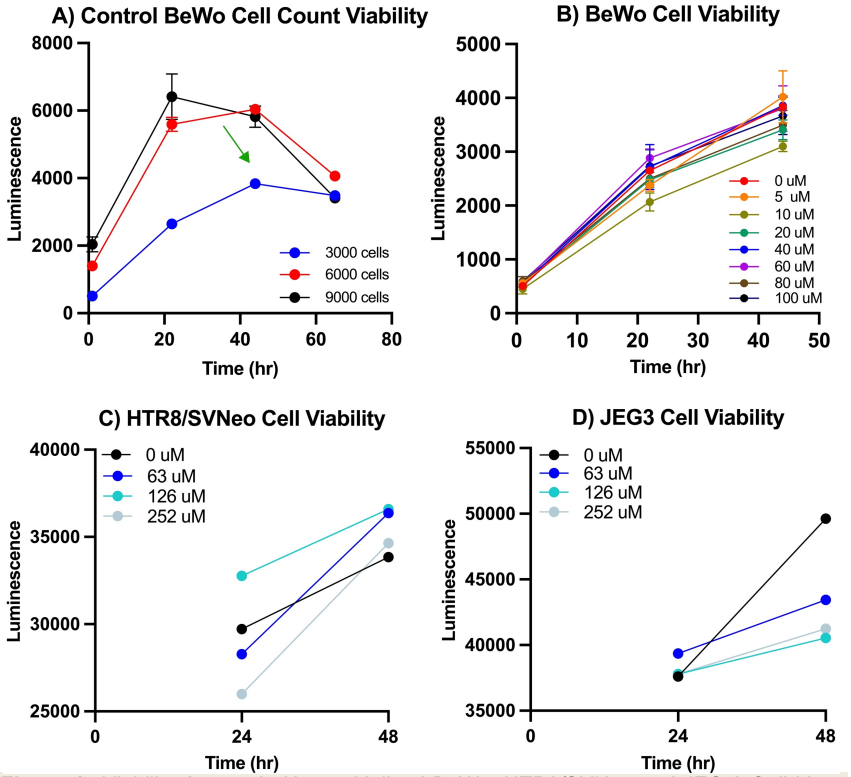

Figure 1. *Viability Assays in Unsyncitialized BeWo, HTR8/SVNeo and JEG-3 Cell Lines to Determine Viability Overtime and establish Ideal GAD Dose.* A) BeWo cell viability in MT viability assay over 22, 44 and 67 hours for various quantities of cells plated (3000, 6000, and 9000). B) Various doses of GAD on cell viability at 24 and 48 hours with MT Assay. C/D) Impact of 4 doses of GAD on HTR8/SVNeo and JEG-3 cell viability at 24 and 48 hours with CellTiter-Glo Assay.

To determine if GAD had a dose-dependent effect on HTR8/SVNeo or JEG-3 cells overtime, we employed the CellTiter-Glo Viability Assay. Each cell type was prepared with 4 doses of GAD (0 um, 63 uM, 126 uM, and 252 uM). These doses were chosen due to the lack of results we saw with the GAD doses used in the BeWo cell viability assay. We anticipated observing a negative slope in cell viability over time as the concentration of GAD increased, indicating a dose-decrease in cell viability. Again, there were no observable dose-dependent or time-dependent effects of GAD on HTR8/SVNeo, or JEG-

3 cell viability (Figure 1C, 1D). It is interesting to note that there is a less pronounced positive slope in JEG-3 cell viability after treatment with 63uM, 226uM and 252uM of GAD compared to the control (Figure 1D). Overall, these results indicate that there is no dose-dependent effect of GAD on Unsyncitialized BeWo, HTR8/SVNeo, JEG-3 cell viability overtime.

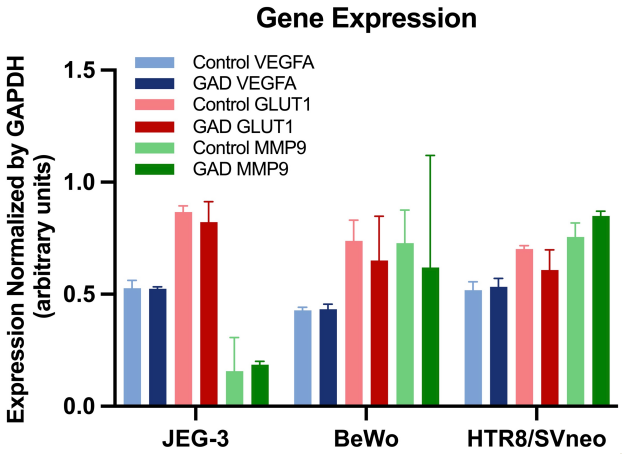

Figure 2. *PCR Quantification of Gene Expression at 48-Hours Post-Treatment with GAD.* GLUT1, MMP9, and VEGF-A genes expression was analyzed in JEG3, BeWo and HTR8/SVNeo trophoblast cell lines 48-hours post PBS (control) or 126uM GAD treatment. Using PCR and gel electrophoresis, gene expression was quantified and normalized by GADPH expression.

GAD Does Not Exhibit an Effect on mRNA Expression in Trophoblast Cell Lines after 48-Hours of Treatment

To assess if GAD interrupts mechanisms important in placental function, within the three trophoblast cell lines (JEG-3, Unsyncitialized BeWo, and HTR8/SVNeo), we performed PCR with VEGF-A, GLUT1 and MMP9. There was no impact of GAD on expression of VEGF-A, GLUT1 or MMP9 in comparison to the controls (Figure 2). It is important to note that to run valid statistical analyses we need more biological replicates, so when we collect more data, these findings might be validated or invalidated with subsequent analyses. The purpose of employing this technique was to assess the impact of GAD on key mechanisms in placental function in trophoblast cells on the level of gene expression, but PCR analysis showed no significant alterations in the expression of VEGF-A, GLUT1, or MMP9 in JEG-3, Unsyncitialized BeWo, and HTR8/SVNeo cells compared to controls.

GAD Does Not Exhibit an Effect on Cytochrome C Expression in Trophoblast Cell Lines after 48-Hours of Treatment

Western Blot analysis was performed on JEG-3, BeWo, and HTR8/SVNeo cell lines after 48 hours of treatment with GAD. We did not observe any effect on the expression of cytochrome C in BeWo and HTR8/SVNeo cell lines; however, GAD does appear to decrease expression of cytochrome C in JEG-3 cells (Figure 3). This result again may not be reliable as there is only one biological replicate. We will need to repeat this experiment to obtain valid statistical analyses. The aim of employing this technique was to assess how GAD affects vital placental mechanisms including cell invasion, glucose transport, and angiogenesis in trophoblast cells on the level of protein expression. Western Blot analysis indicated no alteration in cytochrome C expression in BeWo and HTR8/SVNeo cells post 48-hour GAD treatment, although a decrease was observed in JEG-3 cells.

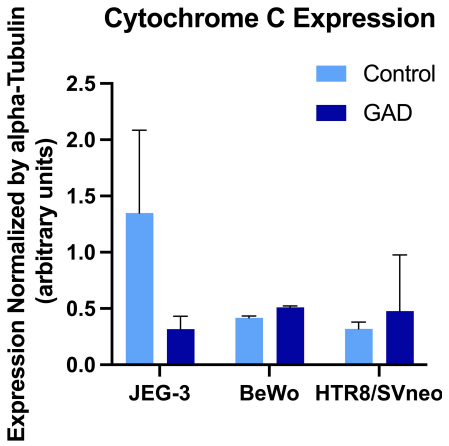

Figure 3. *Western Blot Quantification of Cytochrome C* expression. Performed in JEG-3, BeWo and HTR8/SVNeo trophoblast cell lines 48-Hours after Treatment with PBS (control) or 126uM of GAD.

Discussion/Future Directions

As one of many homeopathic remedies, the mushroom Ganoderma lucidum is known for its anti-tumor effects. Triterpenoids are one of the many active ingredients in this mushroom that show various biological activities associated with their cross-cultural use. GAD is an active triterpenoid associated with specific cytotoxic effects on different cell lines, such as esophageal cancer cells (Shao et al., 2020). Although GAD has been documented for its capability to impede growth, trigger apoptosis, and induce cell cycle arrest in cancer cells, the exact cytotoxic mechanism of GAD remains elusive (Yue et al., 2020).

In this study, we aimed to explore the impact of GAD on representative placental cell lines (BeWo, HTR8/SVNeo, and JEG-3) using various analytical techniques, including viability assays, PCR, and Western Blot. Despite our efforts to establish a dose-dependent effect of GAD (0uM, 5uM, 10uM, 20uM, 40uM, 80uM, 60uM, and 100uM) on BeWo cells, we found no impact over time. This is contradictory to what is seen in previous literature, where two studies were able to establish a dose-dependent impact on cell viability on immortalized cell lines representative of esophageal squamous cell carcinoma (ESCC) (Shao et al., 2020) and human epithelial cells (Yue et al., 2008) with 0uM, 10uM, 20uM, 40uM and 50uM of GAD. Subsequent assessment using the Cell Titer-Glo Assay, with four GAD doses (0uM, 63uM, 126uM, and 252uM), also failed to reveal a dose-dependent effect in either HTR8/SVNeo or JEG-3 cells. Although there appeared to be an impact on JEG-3 cell viability, a clear dose-dependent trend was not evident. While the limited sample size prevents us from conducting valid statistical analyses, the differences in previous literature and our results suggest inherent differences between cell lines used.

Despite not seeing a detrimental impact of GAD on the cell viability of immortalized placenta cell lines, cells can undergo internal disruptions in essential processes in the placenta, such as cell invasion, glucose transport, and angiogenesis, without affecting overall cell viability. MMP9, crucial for extravillous trophoblast invasion and tissue remodeling in pregnancy, when downregulated, can lead to early pregnancy failure; GLUT1, vital for placental glucose transport, when downregulated, may cause gestational diabetes; and VEGF-A, necessary for pregnancy angiogenesis, when downregulated, can result in pre-eclampsia. We did not see an impact of GAD on the expression of any of these genes. Lastly, we used a Western Blot to observe the influence of GAD on the expression of cytochrome C. With a decrease in mitochondria membrane potential, cytochrome C is released from the mitochondria (Shao et al., 2020). We anticipated elevated levels of cytochrome C, suggesting intracellular disruption that leads to apoptosis; however, we did not observe this result; instead, there was no impact of GAD on protein expression. If GAD continues to show no effect on protein expression, we may explore Tubeisomide I, a triterpenoid known to modulate the MAPK pathway, including p38 and ERK1/2, which are crucial for trophoblast cell differentiation (Huang, 2011; Daoud et al., 2005). We have demonstrated that GAD does not cause a dose-dependent decrease in cell viability over time or impact gene or protein expression. Despite our small sample size, it is possible that GAD does not affect any of the pathways explored in this study. However, this research was performed in vitro and is not a complete replacement for in vivo models. In the future, we plan to use primary placenta cells isolated from pregnant female mice to continue exploring the impact of GAD.

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