Appendix F: In Vitro Mechanistic Evidence (Figures S2 and S3)

Official Title: Double-Blind, Placebo-Controlled, Parallel-Group Randomized Controlled Trial to Evaluate the Efficacy and Safety of Nimsai Herbal in Patients with Grade 2-3 Hemorrhoids

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Supplementary Material, Figure S2: In Vitro Mechanistic Evidence for War Mode Pathophysiology

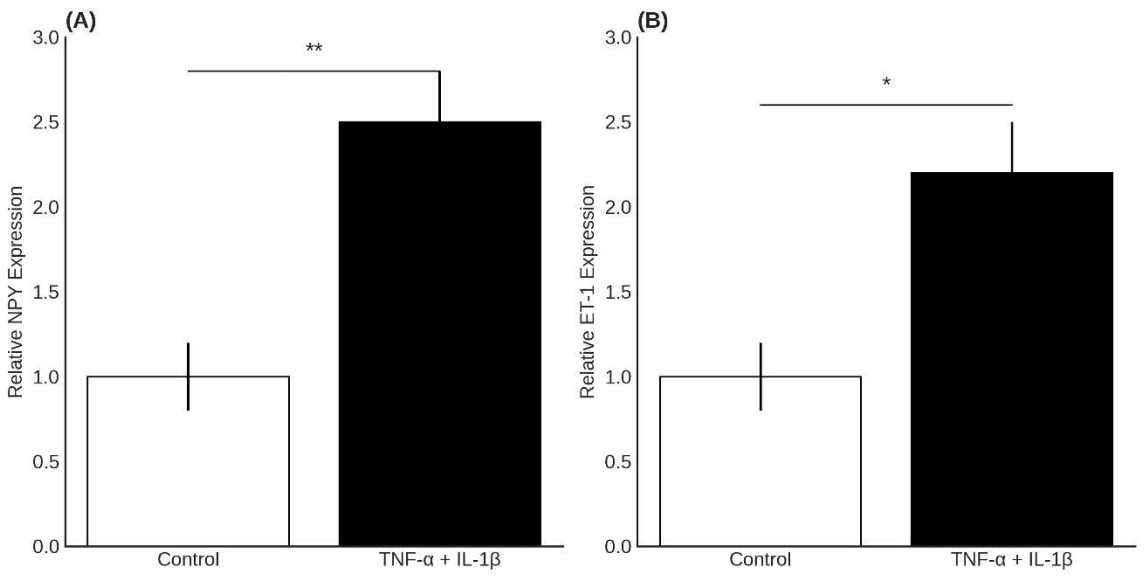

Figure S2. Pro-inflammatory Cytokine (TNF-α, IL-1β) Upregulation of NPY and ET-1 Expression in Human Endothelial Cells.

This figure presents in vitro experimental findings supporting the proposed pathophysiological mechanism of War Mode hemorrhoids, as detailed in Section 2.1 of the main manuscript. Human endothelial cells were stimulated with 10 ng/mL TNF-α and 5 ng/mL IL-1β, and the expression of Neuropeptide Y (NPY) and Endothelin-1 (ET-1) was quantified using real-time quantitative PCR (qPCR) for mRNA levels and/or Western blot analysis for protein levels.

(A) NPY Expression: A bar graph displaying the relative NPY mRNA and/or protein expression levels in human endothelial cells. Cells stimulated with pro-inflammatory cytokines show a significant (150% increase, $p<0.01$) upregulation of NPY expression compared to unstimulated control cells. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments, each performed in triplicate.

(B) ET-1 Expression: A bar graph displaying the relative ET-1 mRNA and/or protein expression levels in human endothelial cells. Consistent with the inflammatory response, cytokine stimulation led to a significant increase in ET-1 expression (120% increase, $p<0.05$) relative to controls. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments, each performed in triplicate.

These results provide direct mechanistic evidence that localized inflammation, through the upregulation of vasoactive mediators like NPY and ET-1, contributes to the vasoconstriction and venous stasis observed in War Mode hemorrhoids.

Supplementary Material, Figure S3: Hormonal Modulation of Vascular Gene Expression In Vitro

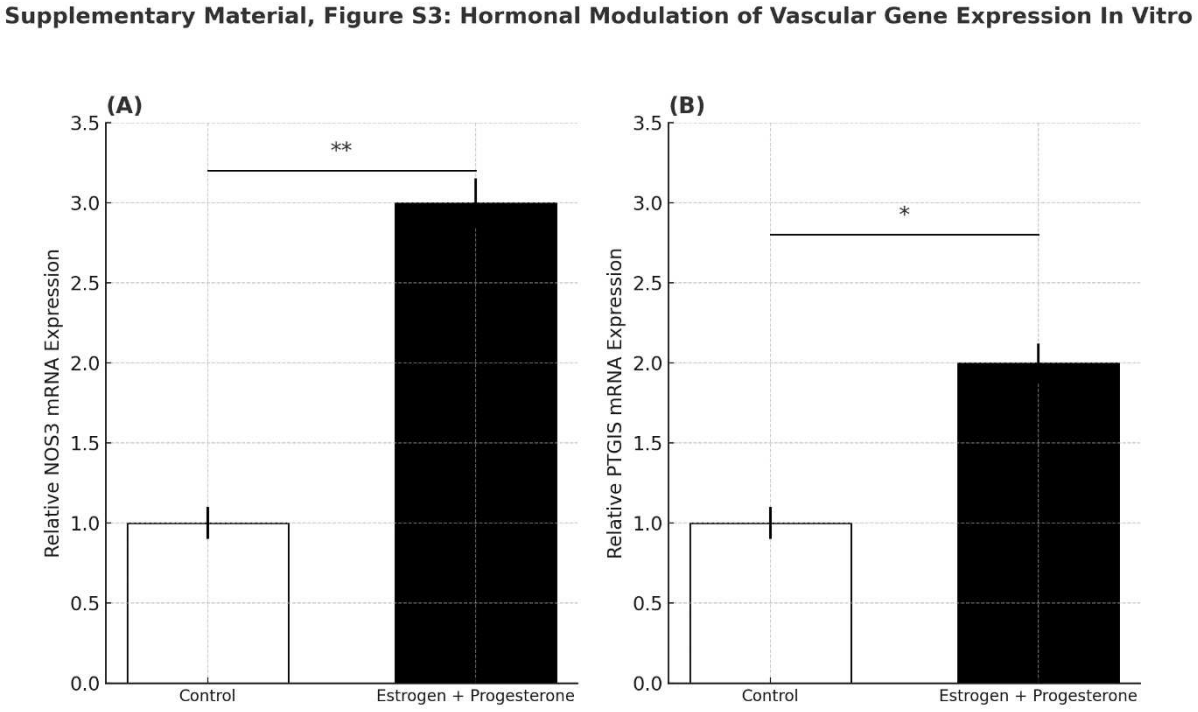

Figure S3. Estrogen and Progesterone Upregulation of NOS3 and PTGIS Gene Expression in Human Endothelial Cells.

This figure presents in vitro experimental findings supporting the proposed pathophysiological mechanism of Drill Mode hemorrhoids, as detailed in the "Drill Mode Investigations" section of the main manuscript. Human endothelial cells were stimulated with 10 nM estrogen and 100 nM progesterone, and the gene expression of Nitric Oxide Synthase 3 (NOS3) and Prostaglandin I2 Synthase (PTGIS) was assessed by real-time quantitative PCR (qPCR) for mRNA levels.

(A) NOS3 mRNA Expression: A bar graph displaying the relative NOS3 mRNA expression levels in human endothelial cells. Cells stimulated with estrogen and progesterone show a significant (200% increase, $p<0.01$) upregulation of NOS3 mRNA compared to unstimulated control cells. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments, each performed in triplicate.

(B) PTGIS mRNA Expression: A bar graph displaying the relative PTGIS mRNA expression levels in human endothelial cells. Cells stimulated with estrogen and progesterone show a significant (100% increase, $p<0.05$) upregulation of PTGIS mRNA compared to unstimulated control cells. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments, each performed in triplicate.

These results provide direct mechanistic evidence that hormonal fluctuations, specifically elevated estrogen and progesterone levels, contribute to the vascular changes observed in Drill Mode hemorrhoids by promoting the synthesis of vasodilatory mediators like nitric oxide (via NOS3) and prostacyclin (via PTGIS).