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An Abstract: Scientific research to examine the physiological effects of InterX® Therapy in humans

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Changes in Lymphocyte Metabolism, Gene Expression and Cytokine production following Transdermal Interactive Neurostimulation: *indicators of Connective Tissue Healing and Anti-inflammatory activity*

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Introduction:

Non-invasive interactive neurostimulation (InterX®) methodologies for treating post-operative pain and facilitating recovery after injury have been anecdotally known to be effective and more recently proven efficacious in clinical trials.^{1,2,3} InterX® is FDA cleared for the treatment of acute and chronic pain,⁵ however a recent clinical trial showed reductions in inflammation in the treated group compared to control*². While the pain relief mechanisms are widely accepted,⁴ the mechanism by which a reduction of inflammation occurs is not well documented. In this study it is hypothesized that changes in lymphocyte metabolism, gene expression and cytokine production following non-invasive interactive neurostimulation may illustrate mechanisms of action related to the body's inflammatory response.

Method:

Treatment protocol: A multidisciplinary approach was used to examine the effect of transdermal interactive neurostimulation with the InterX® 5002⁵ device on lymphocyte metabolic function, gene expression and cytokine production. Blood was drawn from 4 healthy adults (2M/2F) before and 20 minutes following a treatment session. Treatments consisted of 10 minutes of treatment using 480 pulse/second stimulation on the lateral elbow of the arm from which blood was drawn and 10 minutes with a 90-360 variable pulse/second stimulation over the corresponding spine root.

Sample preparation: Venous Blood (7 mL) was drawn directly into PAXGene tubes (Quiagen, Valencia CA) and processed according to the manufacturers directions. This yielded lymphocyte-derived Ribonucleic Acid (RNA), which was sent to a gene-chip analysis core at Cincinnati Children's Hospital Medical Center. Intact fresh Lymphocytes were isolated from a further 5-7mL of venous blood (collected into Vacutainer tubes containing citrate as an anti-coagulant (BD,NJ)) using a density gradient protocol (Lymphoprep, Grenier-Bio-one, Germany) following the manufacturers directions. Aliquots of cells were set aside for protein analysis and flash-frozen in N (I) for cytokine analysis and the remainder were diluted with respiration medium (Mir05, Oroboros, Innsbruck, Austria) for metabolic analysis. Protein was quantified using Pierce BCA protocol (Pierce, Rockford, IL).

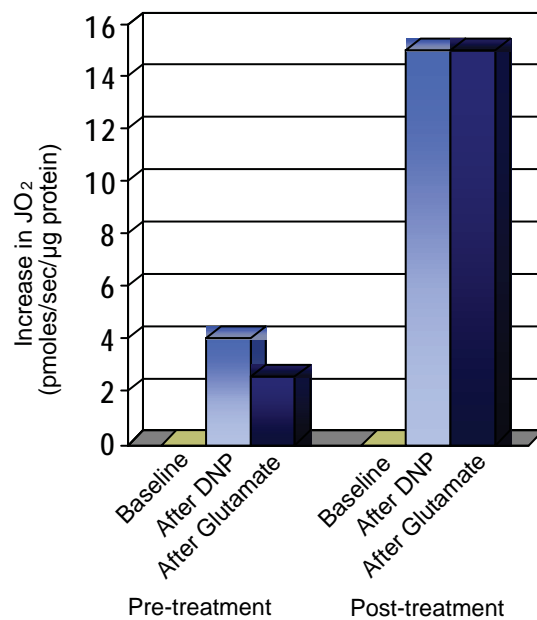
Results:

Lymphocyte metabolism: Lymphocytes were allowed to respire at 37deg C in the chamber of a high-resolution respirometry system (Oroboros, Innsbruck, Austria). A baseline respiration rate was obtained and then a 10mM glutamate system was added and the respiration rate calculated for that stimulation. The cells were then stimulated by dinitrophenol (DNP) (final concentration of 10 nM). The cells were once again allowed to respire until they reached a steady state.

As seen in Fig.1, there was no significant difference in baseline respiration rates between pre- and post-treatment. These data suggest that metabolic activity of mitochondrial containing blood cells is unaffected by InterX[®] treatment. However, the InterX[®] treatment produced a significant improvement of the cells ability to respond to physiological and pathological stimuli (Glutamate and DNP respectively). The glutamate induced significantly greater relative increase in respiration over baseline post- treatment compared to pre-treatment. This may be indicative of an improved/ enhanced ability of these cells to respond to stimuli when necessary which could result in enhanced healing ability post injury.

Gene expression: Both up- and downregulated gene activity was seen in this study. Genomic changes were considered significant if they were seen in all 4 subjects and were changed by 2 fold or more post-treatment. As expected there were up-regulated genes corresponding to mitochondrial stimulation, which supports the results from the lymphocyte study (COX 2, ATP synthase). Neuron-specific genes were also upregulated, indicating that transdermal electrical neuromodulation does indeed affect nervous tissue (Neuronal pentraxin). Of particular interest in the upregulated gene set is LOX 4 which is specifically related to cartilage regeneration and repair. Lysyl Oxidase-Like enzyme IV (LOXL4) is a copper-dependent amine oxidase that is responsible for the catalysis of cross-link formation in collagen and elastin. LOX4 has also been shown to inhibit MMP2 (matrix metalloproteinase II) thus having a net pro-extracellular matrix formation effect. Upregulation of this gene product is therefore desirable following injury.⁷

Figure.1 Lymphocyte Metabolism Response



Increase in JO₂ over baseline in mononuclear leukocytes from four donors pre-treatment (control) and post-treatment (experimental) transdermal stimulation

Figure.2 Upregulated Genes

GENE	Protein	p value	function
SM00159	Pentraxin	0.000374	Increase in serum upon infection or trauma. Cytokine-induced, acute innate immune response molecules.
IPR001759	Neuronal pentraxin receptor	0.000522	NEURON ASSOCIATED NPTXR
PF00354	Pentraxin	0.000522	IBID
PF02145	Rap/Ran GTPase AP	0.000374	Cytoskeleton arrangement
PS50085	Rap/Ran GTPase AP	0.000522	actin-mediated motility
IPR000331	Rap/Ran GTPase AP	0.000522	NEURON ASSOCIATED
117029	COX 2	0.000046	Mitochondrial gene
114457	ATP synthase	0.002014	Mitochondrial protein
chr10q24	LOXL 4. Lysyl oxidase-like 4	0.000167	Cartilage and connective tissue generation and repair enzyme.

Downregulated genes include those responsible for activation, adhesion and recruitment of inflammatory cells. This may be important in the putative anti-inflammatory effects of the InterX[®] device.

Figure.3 Downregulated Genes

GENE	Protein	p value	function
16160	ANT	0.000082	Adenine nucleotide transporter
STARCH	AMY1A,AMY2A,AMY1C,AMY	0.000194	Amylases
METABOLISM	1B,		
hsa04514	HLA-DOA, HLA-DQB1	0.000217	Cell adhesion molecules
h_cxcr4Pathway	CXC chemokine	0.000394	immune cell attractant
PD000050	MHC_I	0.000001	I CLASS MHC ANTIGEN TRANSMEMBRANE
IPR001039			PRECURSOR SIGNAL CHAIN HISTOCOMPATIBILITY
PF00129			ALPHA
IPR010579			
PF07654	C1-set	0.000075	Immunoglobulin C-1 set domain

Cytokine Production: Flash-frozen lymphocytes were thawed on ice and loaded directly into multiplex cytokine assay plates (Milliplex, Anderson, CA). The plates were pre-coated with antibodies to TNF- α , VEGF, IL-1 α , IL-1 β and IL-6. The ELISA kit was processed following the manufacturers directions and the plate was read using Luminex xMap software with a Biorad Bioplex reader.

Significant increases in TNF- α , IL-1 β and IL-6 were observed. All three of these cytokines are associated with acceleration of wound and connective tissue healing^{8,9,10} Decreased levels of VEGF and IL-1 α were seen; this is consistent with reduced inflammation and support of wound healing processes.^{11,12} Total protein content of the lymphocyte preparations were not significantly different between pre- and post-treatment samples, meaning these results were not due to proliferation of white cells, rather activation.

Discussion: We believe that the sum of these results is that the lymphocytes post InterX[®] treatment may be better able to respond to stimuli when necessary which could result in enhanced healing ability post injury. This is concluded based on a lack of change in baseline oxygen consumption but greater responses to external stimuli. Further, the genomic profile seen after just 20 minutes is indicative of an acute response that would infer improved ability of these cells to respond to injury types of stimuli. The total protein content of the lymphocyte preparations were not significantly different between pre- and post-treatment samples, meaning these results were not due to proliferation of white cells, rather activation of those cells. Therefore it seems less likely that the lymphocytes would be capriciously participating in inflammatory responses, but able to respond better when needed. Further experiments are needed to determine whether or not this actually will improve wound healing.

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