# Changes in Lymphocyte Metabolism, Gene Expression and Cytokine production following Transdermal Interactive Neurostimulation (InterX®) : *Indicators of Connective Tissue Healing and Anti-inflammatory Activity*

*Joseph F. Clark, ATC, PhD.*[1] *Paul J Magee (Neuro Resource Group, Plano. TX). Gail Pyne-Geithman*[2] *D Phil*
*Department of Neurology,*[1] *Department of Neurosurgery* [2]*, University of Cincinnati, OH. 45267-0536, USA.*

*Presented as a poster at the 13th World Congress on Pain, Montreal. Aug 29th - Sept. 2nd, 2010. PW 253*

## Introduction:

Non-invasive interactive neurostimulation (InterX) has shown to be clinically effective for a range of acute and chronic conditions[1,2,3] and in experimental pain research[4]. InterX also demonstrated reduced inflammation in patients following ankle surgery [2] . While mechanisms for pain relief for this type of therapy are well understood [5,6], it has previously been demonstrated that trancutaneous electrical stimulation does not affect the inflammatory response[7]. This study was designed to research some of the underlying mechanisms that may influence the anti-inflammatory effects of InterX by studying lymphocyte metabolism responses, gene expression and the production of cytokines.

## Hypothesis:

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 using the InterX 5002 device manufactured by InterX Technologies, Richardson, Texas to measure lymphocyte metabolic function, gene expression and cytokine production. Blood was drawn from 4 healthy adults (2M/2F) prior to and 20 minutes following a treatment with the InterX 5002 neurostimulation device. Treatment applications consisted of 10 minutes of treatment using 480 pulse/second stimulation on the lateral elbow of the arm from which blood was drawn (Fig 1). The operator used the 5002 device following the Scan and Target protocol to identify areas of low impedence and then deliver high amplitude stimulation to the cutaneous nerves at each of the low impedance sites. A further 10 minutes of stimulation was delivered to the corresponding spine root (C3-C7) using a 90-360 variable pusle/second stimulation delivered through the InterX 4X4 Flexible Array. The configuration of the Flexible Array electrodes ensures that stimulation is drawn to the points of low impedance which are optimal treatment points for neurostimulation[8-11].

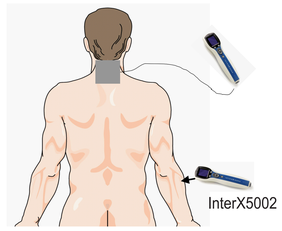


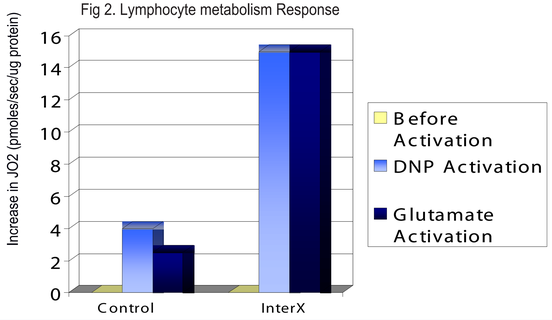

Fig 1. InterX treatment areas

***Sample preparation:*** Venous Blood (7mL) was drawn directly into PAXGene tubes (Quiagen, Valencia CA) and processed according to the manufacturers instructions. This yielded lymphocyte-derived Ribonucleic Acid (RNA), which was sent to a gene-chip analysis core at the Cincinnati Children's 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 (l) 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).

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

***Cytokine production:*** Flash-frozen lymphocytes were thawed on ice and loaded directly into mutilplex cytokine assay plates (Milliplex, Anderson, CA). The plates were pre-coated with antibodies to TNF-$\alpha$, IL-1$\alpha$ , VEGF, IL-1$\beta$ and IL-6. The ELISA kit was processed following the manufacturer's directions and the plate was read using Luminex xMap software with Biorad Bioplex reader.

Fig 2. Lymphocyte metabolism Response




## Results:

As seen in Fig.2, there was no significant difference between baseline respiration rates pre- and post-treatment. These data suggest that metabolic activity of mitachondrial 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 pathalogical stimuli, p<.05 (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 four subjects and were changed by two-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 InterX transdermal electrical stimulation does indeed affect nervous tissue (Neuronal pentraxin). Of particular interest in the upregulated gene set is LOXL4 (Table 1) which is specfically related to cartilage regeneration and repair. Lysyl Oxidase-Like enzyme IV (LOXL4) is a copper dependent amine oxida that is responsible for the catalysis of cross-link formation in collagen and elastin. LOXL4 has also been shown to inhibit MMP2 (matrix mertalloproteinase II), thus having a net pro-extracellular matrix formation effect. Upregulation of this gene is therefore desirable following injury.

Adenosine Triphosphate (ATP) was released in response to InterX stimulation. Once released, ATP acts as a transmitter that binds to purinergic receptors, including P2X and P2Y receptors[12,13] . ATP cannot be transported back into the cell but is rapidly degraded to adenosine by several ectonucleotidases before re-uptake[13] .Thus adenosine acts as an analgesic agent that supresses pain through Gi-coupled A1-adenosine receptors[14-16] . Adenosine also has anti-inflammatory properties[17] .

Down regulated genes (Table 2) include those responsible for activation, adhesion and recruitment of inflammatory cells. This may be important in the putative anti-inflammatory effects of the InterX neurostimulation device.

***Cytokine production:***

Cytokines known to be associated with, or essential for connective tissue, skin and wound healing were significantly (p<0.05) increased following InterX treatment in all 4 subjects

Cytokines known to elicit oxidative metabolism in lymphocytes were significantly (p<0.05) increased following InterX treatment in all 4 subjects. This supports the white cell respiration data.

Significant increases in TNF-$\alpha$, IL-1$\beta$ and IL-6 were observed. All three of these cytokines are associated with acceleration of wound and connective tissue healing. Decreased levels of VEGF and IL-1$\alpha$ were seen; this is consistent with reduced inflammation and support of the wound healing process. Total protein content of the lymphocyte preparations were not significantly different between pre- and post-treatment samples, meaning that these results were not due to proliferation of white cells and rather due to activation.

**TABLE 1:** Genes significantly *upregulated* in all 4 subjects after treatment with the InterX device.

| GENE | Protein | p value | function | reference |
|---|---|---|---|---|
| SM00159 | Pentraxin | 0.000374 | *Increase in serum upon infection or trauma. Cytokine induced, acute innate immune response molecules.* | Goodman 1996[6] |
| IPR001759 | Neuronal pentraxin receptor | 0.000522 | *NEURON ASSOCIATED NPTXR* | Dunham 1999[7] |
| PF00354 | Pentraxin | 0.000522 | *IBID* | Goodman 1996[6] |
| PF02145 | Rap/Ran GTPase AP | 0.000374 | *Cytoskeleton arrangement* | Lundquist 2006[8] |
| PS50085 | Rap/Ran GTPase AP | 0.000522 | *actin - mediated motility* | Lundquist 2006[8] |
| IPR000331 | Rap/Ran GTPase AP | 0.000522 | *NEURON ASSOCIATED* | Nagase 1999[9] |
| 117029 | COX 2 | 0.000046 | *Mitochondrial gene* | Nelson 1993[10] |
| 114457 | ATP synthase | 0.002014 | *Mitochondrial protein* | Falk 1988[11] |
| chr10q24 | LOXL 4. Lysyl oxidase-like 4 | 0.000167 | *Cartilage and connective tissue generation and repair enzyme.* | Maki 2005 [1 2] |

**TABLE 2:** Genes significantly *downregulated* in all 4 subjects after treatment with the InterX device.

| GENE | Protein | p value | function | reference |
|---|---|---|---|---|
| 16160 | ANT | 0.000082 | *Adenine nucleotide transporter* | Schuster 1993[15] |
| STARCH METABOLISM | AMY1A,AMY2A,AMY1C,A MY1B, | 0.000194 | *Amylases* | Boer 1986[16] |
| hsa04514 | HLA-DOA, HLA-DQB1 | 0.000217 | *Cell adhesion molecules* | Campagna 2008[17] |
| h_cxcr4Pathway | CXC chemokine | 0.000394 | *immune cell attractant* | Campagna 2008[17] |
| PD000050 IPR001039 PF00129 IPR010579 | MHC_I | 0.000001 | *I CLASS MHC ANTIGEN TRANSMEMBRANE PRECURSOR SIGNAL CHAIN HISTOCOMPATIBILITY ALPHA* | Madden 1992[18] |
| PF07654 | C1-set | 0.000075 | *Immunoglobulin C-1 set domain* | Greenberg 1996[19] |

## Conclusion:

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 that these results were not due to proliferation of white cells, but rather due to the 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 warranted to determine whether or not this actually will improve wound healing.

## References:

1. **Gorodetskyi I.G., et al.** Non-invasive interactive neurostimulation in the post-operative recovery of patients with a trochanteric fracture of the femur. *J Bone Joint Surg (Br) 2007;89-B:1488-94*
2. **Gorodetskyi I.G., et al:** The effects of non-invasive interactive neurostimulation on pain and edema during post-surgical rehabilitation following internal fixation of unstable bi-malleolar ankle fractures. J Foot Ankle Surg 2010; 49:432-437
3. **Maale G, Gamez M.** The effects of a handheld, cutaneous, portable neurostimulator using two concentric electrodes with signals that are damped, bi-phasic oscillatery waveform which use the skin as a conduit in patients with chronic severe pain from large orthopaedic procedures. *18th Annual Symposium International Society for Technology in Anthroplasty,* 2005:143
4. ***K. Gilbey; J. McKenna; E. Gardner; M.K. Hoeger Bement; M Johnson; K.A. Sluka; A.G. Leacock; D.M. Walsh.*** A Preliminary Investigation of the Hypoalegesic Effects of InterX on Experimental Pain: *APTA CSM 2010, February 16-20, 2010, presented as a poster*
5. **Sluka KA, Walsh D.** Transcutaneous electrical nerve stimulation: basic science mechanisms and clinical effectiveness. *J Pain 2003;4:109-21*
6. **Han J S,** Acupuncture: neuropeptide release produced by electrical stimulation of different frequencies. Trends in Neurosciences, Vol. 26, No.1, January 2003
7. **Resende M.A., et al,** Local transcutaneous electrical stimulation (TENS) effects in experimental inflammatory edema and pain, European Journal of Pharmacology 504 (2004) 217– 222
8. **Walsh D.** Transcutaneous electrical nerve stimulation. In Acupuncture and Related Techniques in Physical Therapy. Eds. Hopwood V, Lovesey M, Mokone S. New York: Churchill Livingston: 1997: 111 – 118.
9. **Melzack R:** Prolonged relief of pain by brief, intense transcutaneous somatic stimulation. Pain. 1975;1: 357-373.
10. **Shultz S.P., et al,** The evaluation of Electrodermal Properties in the Identification of Myofascial Trigger Points. Arch Phys Med Rehabil, Vol 88, June 2007
11. **Colbert A.P., et al,** Skin Impedance Measurements for Acupuncture Research: Development of a Continuous Recording System. eCAM 2008 5(4):443-450; doi:10.1093/ecam/nem060
12. **Abbracchio, M.P., Burnstock, G., Verkhratsky, A. & Zimmermann, H. Purinergic** signaling in the nervous system: an overview. Trends Neurosci. 32, 19–29 (2009).
13. **Burnstock, G.** Physiology and pathophysiology of purinergic neurotransmission. Physiol. Rev. 87, 659–797 (2007).
14. **Sjölund, K.F., Segerdahl, M. & Sollevi, A.** Adenosine reduces secondary hyperalgesia in two human models of cutaneous inflammatory pain. Anesth. Analg. 88, 605–610 (1999).
15. **Maione, S. et al.** The antinociceptive effect of 2-chloro-2'-C-methyl-N6-cyclopentyladenosine (2'-Me-CCPA), a highly selective adenosine A1 receptor agonist, in the rat. Pain 131, 281–292 (2007).
16. **Sawynok, J., et al.** Peripheral antinociceptive effect of an adenosine kinase inhibitor, with augmentation by an adenosine deaminase inhibitor, in the rat formalin test. Pain 74, 75–81 (1998).
17. **Fredholm, B.B.** Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. 14, 1315–1323 (2007).