INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY Vol. 25, no. 2, 317-323 (2012)

EDITORIAL

A CASE SERIES OF A LUTEOLIN FORMULATION (NEUROPROTEK®) IN CHILDREN WITH AUTISM SPECTRUM DISORDERS

T.C. THEOHARIDES[1,2,3,4], S. ASADI[1,5] and S. PANAGIOTIDOU[1,6]

[1]Laboratory of Molecular Immunopharmacology and Drug Discovery, Department of Molecular Physiology and Pharmacology, and Departments of [2]Biochemistry,[3]Internal Medicine, and [4]Psychiatry, Tufts University School of Medicine and Tufts Medical Center, Boston, MA, USA; [5]Department of Pharmacy, Tufts Medical Center, Boston, MA, USA; [6]Department of Geography and Environment, Boston University, Boston, MA, USA

Received December 13, 2011 – Accepted March 5, 2012

There has been an impressive, little understood increase in cases of Autism Spectrum Disorders (ASD). The lack of any distinctive pathogenetic mechanism has hampered the development of any effective treatments. Increasing evidence indicates oxidative stress, brain inflammation, gastrointestinal (GI) dysfunction and allergic symptoms may be present in ASD patients. The flavone luteolin has anti-oxidant, anti-flammatory, anti-allergy and neuroprotective properties. Given these findings, a dietary supplement was developed with a unique mixture of luteolin with the related flavonoids quercetin and rutin in a liposomal formulation of olive kernel oil (OKO), which increases their absorption. Results are presented for children with ASD (n=37, 4-14 years old) who had not obtained any benefit from multiple other regimens and who used this formulation for at least 4 months. GI and allergy symptoms improved in about 75% of children, eye contact and attention in 50%, social interaction in 25% and resumption of speech in about 10%. There were no adverse effects. Even though these results represent an uncontrolled open case series, they are encouraging because they suggest good tolerability and potential effectiveness.

Autism Spectrum Disorders (ASD) are pervasive developmental disorders, diagnosed in early childhood (1) that have increased more than 10-fold during the last decade to approximately 1/100 live births (2). They are characterized by varying degrees of defective communication and social skills, cognitive, learning and sensory defects, as well as repetitive and stereotypic behaviors (1, 2). The lack of any definitive pathogenesis has prevented the development of effective treatments for the core symptoms of ASD (3, 4). In fact, recent studies have shown that use of selective serotonin reuptake inhibitor (SSRI) antidepressants (5) and some antipsychotics (6) may actually worsen ASD symptoms and cause the onset of adverse effects (7). Consequently there is a great need for novel approaches to address ASD.

Role of mast cells

Mast cells are critical for the development of allergies (8), inflammation (9), and autism (10) because they can secrete a plethora of vasoactive and inflammatory mediators that include histamine, kinins, and tryptase (preformed), as well as

Key words: autism, brain, flavonoids, inflammation, luteolin, mast cells, treatment

Mailing address: T.C. Theoharides, M.S., Ph.D., M.D.
Department of Molecular Physiology and Pharmacology,
Tufts University School of Medicine,
136 Harrison Avenue,
Boston, MA 02111, USA
Tel: +1 617 636-6866 Fax: +1 617 636-2456
e-mail: theoharis.theoharides@tufts.edu

0394-6320 (2012)

chemokines, and cytokines, such as IL-6, IL-8, and TNF (9). Mast cells are the only immune cells that store preformed TNF (11). Neuro-immunoendocrine activation of mast cells in animal models can lead to disruption of both the gut-blood and blood-brain barriers (BBB) (12). It is of particular importance that mast cells are also activated by neuropeptides often secreted during stress, such as neurotensin (9), which has been shown to be elevated in the serum of young children with autism (13). Acute stress increased serum IL-6 levels, a response that was entirely dependent on mast cells (14).

Brain inflammation in ASD

Increasing evidence indicates that there is some immune dysfunction in ASD (15). Postmortem brains of ASD patients had evidence of microglial activation and inflammation (16). CSF levels of TNF were significantly increased in autistic children (17), while brains of ASD patients had elevated expression of IL-6 (18). Maternal viral infection or viral poly (I:C) injection in pregnant mice increased circulating IL-6 levels that crossed the placenta at midgestation and contributed to fetal brain injury and autistic-like behavior (19).

Numerous reports from patients and recent studies indicate the presence of "allergic-like" symptoms in children with ASD (20-22), implying mast cell activation. A preliminary retrospective study also reported much higher prevalence of ASD (1/10 children) in mastocytosis patients characterized by more and activated mast cells, than the general population (1/100 children) (23). It is interesting that mastocytosis severity was shown to correlate with increased serum levels of IL-6 (24).

Effect of flavonoids

Some naturally occurring flavonoids have potent anti-oxidant, and anti-inflammatory activities (25). They can also inhibit histamine, IL-6, IL-8, TNF, and tryptase release from human mast cells (26). In view of the fact that ASD has been associated with oxidative stress (27), increased brain IL-6 expression and mast cell release of IL-6, especially in response to stress, we compared the ability of the flavone luteolin to that of its structurally-related flavone quercetin to inhibit IL-6 production from human cultured mast cells (Table I). We report that

Table I. *Effect of luteolin, quercetin and different oils on IL-6 release from HMC-1 cells+.*

Conditions	IL-6 (pg/10 ^{6} cells)
Basal	25
IL-1 (50 ng/ml)	632
Corn oil + IL-1	680
Olive oil + IL-1	510
OKO[#]	393
DMSO (0.1%)	423
Quercetin (10 μM in DMSO)+ IL-1	280
Luteolin (10 μM in DMSO)+ IL-1	149
Luteolin (10 μM in OKO)+ IL-1	89

[+]Human leukemic cells (HMC-1) were pre-incubated with the compounds shown for 10 min at 37 C before stimulation with IL-1 (50 ng/ml) for 12 h. IL-6 was measured by ELISA (R&D systems, Minneapolis, MN).
[#] OKO (Olive Kernel Oil)

Table II. *Benefits of luteolin relevant to ASD.*

Properties	Benefits
Flavone (4 phenolic groups)	Crosses blood-brain-barrier (BBB)
Anti-oxidant	Reduces brain oxidative stress
Anti-inflammatory	Reduces gut and brain inflammation
Mast cell inhibitor	Reduces allergic reactions
Microglia inhibitor	Reduces brain inflammation
Anti-cytokine	Inhibits IL-6 production
Stabilizes blood vessels	Protects against BBB disruption
Mimics BDNF [#]	Promotes neuronal recovery
Is neuroprotective	Protects neuronal damage
Reverses autistic behavior in mice	Has beneficial effect *in vitro*

[#] *BDNF (Brain-derived neurothrophic factor)*

flavonoids were best dissolved in olive kernel oil (OKO) and luteolin was more potent than quercetin inhibiting IL-6 release (Table I).

Effects of luteolin relevant to ASD

The beneficial actions of luteolin relevant to ASD are listed in Table II. Luteolin inhibits production of IL-6 from microglia (28), and astrocytes (29). Luteolin can also induce anti-inflammatory changes and neuroprotective changes in glial cells (30). Luteolin also inhibits mast cell-dependent stimulation of activated T cells that participate in autoimmune diseases (31) and inhibits maternal IL-6-induced autism-like behavioral deficits in social interaction in mice (32). Luteolin also blocks mercury-induced cytokine release from human mast cells (33). Luteolin, and its structurally related, quercetin, inhibit histamine, leukotrienes and prostaglandin D_2 release from human cultured mast cells (34). Quercetin also protected against swimming stress-induced increase in serum lipid hydroperoxide levels in rats (35), and reversed acute stress-induced autistic-like behavior and low brain glutathione levels in mice (36).

NeuroProtek®

We designed a dietary flavonoid formulation to address the pathologic processes apparently present in ASD described above. This formulation (NeuroProtek®) is made by a Good Manufacturing Practices (GMP)-certified facility under contract from Algonot, LLC (www.algonot.com). NeuroProtek® has a "Certificate of Free Export" from the Food and Drug Administration (FDA). NeuroProtek® contains mainly luteolin (100 mg, from chamomile >95% pure), quercetin (70 mg, from Saphora, >95% pure), which will inhibit mast cell and act as "decoy" allowing more luteolin to escape metabolism and reach the brain, and the quercetin glycoside rutin (30 mg, from Saphora, >95% pure), which gets cleaved by intestinal bacteria and will reduce gut inflammation. Unfortunately, flavonoids are poorly absorbed after oral administration and

Table III. *Apparent benefits of Neuroprotek® in ASD*.*

Symptoms	Approximate improvement of children
Stool color, shape, smell	28/37
"Allergic problems"	19/37
Eye contact	15/37
Retention of learned aspects	15/37
Social interaction	14/37
Attention to directions	12/37
Speaking words/sentences	4/37
Hyperactivity/Aggression	-

** (n= 37, 4-14 years old; 29 boys and 8 girls)*

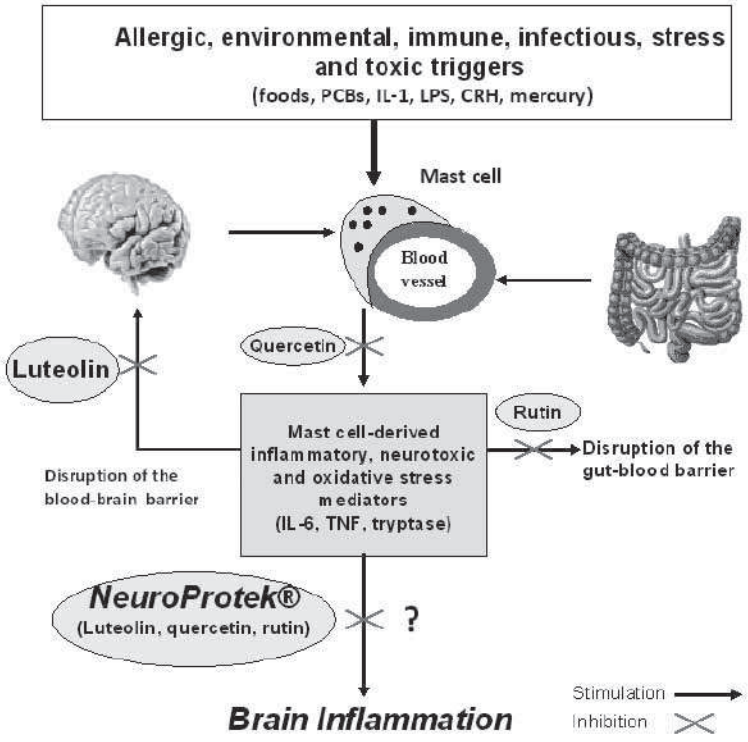

Fig. 1. *Diagrammatic representation of the sites of beneficial action of the flavonoids in NeuroProtek®. Rutin will be generated and act primarily in the gut and reduce gut-inflammation and gut-blood barrier disruption. Quercetin will act in all tissues and mostly in the skin to reduce "allergic-like" reactions, while luteolin will reduce brain inflammation and BBB permeability.*

undergo significant liver metabolism (37) limiting their systemic availability. Increased absorption of luteolin and quercetin is achieved by formulation in microspheres (liposomes) by mixing in OKO of low oleic acid and water content (< 0.1%).

Each soft gel capsule contains 200 mg total flavonoid. We recommended at least 2 capsules/20 kg weight, or at least 400 mg total flavonoid. However, we may be underdosing. For instance, luteolin at 10 μM (necessary for 80% mast cell inhibition) is 3 mg per 1 liter=1 kg, or 60 mg per 20 kg body weight. If we were to assume that our body is one compartment with equal distribution and maximal 10% absorption of luteolin, we should be administering 10x60 mg=600 mg/20 kg body weight, or 3 capsules/20 kg.

Apparent beneficial effects of NeuroProtek®

Data were obtained from responses submitted by the parents of children with ASD (n=37, 4-14 years old, 29 boys and 8 girls) from throughout the USA (Table III). On average, patients (75%) reported significant improvement in their bowel color, form and habits within 2-3 weeks. "Allergic-like" symptoms, especially in the skin, were also significantly reduced in most of the children. Eye contact and increased attention to directions improved in about 50% of patients. As many as 30-50% of patients showed significant improvement in retained learned tasks and social interactions. Surprisingly, about 10% of children started speaking words or sentences. There was no apparent improvement in hyperactivity or aggressiveness. In fact, in 1-2 children who were apparently "intolerant to phenols", hyperactivity may have increased temporarily and decreased when the dose was lowered after two weeks.

The apparent benifit of NeuroProtek® could be through reduction of brain and gut inflammation and may do so through inhibition of different processes, many of which involve mast cells (Fig. 1).

CONCLUSION

The present results are encouraging, since they suggest that a liposomal formulation of luteolin and quercetin, which are safe (38, 39), are well tolerated and can have a positive impact. It is not apparent which ASD children will be helped most and to what extent. The results presented here are obviously limited by the lack of any specific psychometric instruments and an independent assessment by an external evaluator. A clinical study using NeuroProtek® with various behavioral and biomarker end points in ASD children is ongoing in Greece and will be reported in the Fall of 2012. Moreover, a new formulation with reduced phenol content is available. It would also be interesting to assess the effect of NeuroProtek® together with other anti-inflammatory, anti-allergic and neuro-protective drugs such as the newer antidepressants (40).

ACKNOWLEDGEMENTS

Some aspects of the *in vitro* work described above were funded by the Autism Collaborative, National Autism Association, Safe Minds and Theta Biomedical Consulting and Development Co., Inc. (Brookline, MA, USA).

DISCLOSURES: TCT is the inventor of US patents No. 6,624,148; 6,689,748; 6,984,667, and EPO 1365777, which cover methods and compositions of flavonoids in inflammation, US patents No 7,906,153 and 12/861,152 (allowed) for treatment of neuro-inflammatory conditions, as well as US patent applications No. 12/534,571 and No.13/009,282 for the diagnosis and treatment of ASD. TCT is also the inventor of NeuroProtek®, with US trademark No. 3,225,924. Theta Biomedical Consulting and Development Co., Inc. and Tufts University have entered into an agreement for the intellectual property rights and distribution of NeuroProtek®. A portion of any profits from NeuroProtek® will be donated to autism-related charities.

REFERENCES

1. Johnson CP, Myers SM. Identification and evaluation of children with autism spectrum disorders. Pediatrics 2007; 120(5):1183-215.
2. Gurney JG, McPheeters ML, Davis MM. Parental report of health conditions and health care use among children with and without autism: National Survey of Children's Health. Arch Pediatr Adolesc Med 2006; 160(8):825-30.

3. Broadstock M, Doughty C, Eggleston M. Systematic review of the effectiveness of pharmacological treatments for adolescents and adults with autism spectrum disorder. Autism 2007; 11(4):335-48.
4. Rogers SJ, Vismara LA. Evidence-based comprehensive treatments for early autism. J Clin Child Adolesc Psychol 2008; 37(1):8-38.
5. King BH, Hollander E, Sikich L, et al. Lack of efficacy of citalopram in children with autism spectrum disorders and high levels of repetitive behavior: citalopram ineffective in children with autism. Arch Gen Psychiatry 2009; 66(6):583-90.
6. McPheeters ML, Warren Z, Sathe N, et al. A systematic review of medical treatments for children with autism spectrum disorders. Pediatrics 2011; 127(5):e1312-21.
7. Bersani G, Gherardelli S, Clemente R, et al. Neurologic soft signs in schizophrenic patients treated with conventional and atypical antipsychotics. J Clin Psychopharmacol 2005; 25(4):372-5.
8. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature 2008; 454(7203):445-54.
9. Theoharides TC, Alysandratos KD, Angelidou A, et al. Mast cells and inflammation. Biochim Biophys Acta 2010; 1822(1):21-33.
10. Theoharides TC, Angelidou A, Alysandratos KD, et al. Mast cell activation and autism. Biochim Biophys Acta 2012; 1822(1):34-41.
11. Saggini A, Tripodi D, Maccauro G, et al. Tumor necrosis factor-alpha and mast cells. Eur J Inflamm 2011; 9(1):17-22.
12. Theoharides TC, Doyle R. Autism, gut-blood-brain barrier and mast cells. J Clin Psychopharmacol 2008; 28(5):479-83.
13. Angelidou A, Francis K, Vasiadi M, et al. Neurotensin is increased in serum of young children with autistic disorder. J Neuroinflammation 2010; 7:48.
14. Huang M, Pang X, Karalis K, Theoharides TC. Stress-induced interleukin-6 release in mice is mast cell-dependent and more pronounced in Apolipoprotein E knockout mice. Cardiovasc Res 2003; 59(1):241-9.
15. Ashwood P, Wills S, Van de Water J. The immune response in autism: a new frontier for autism research. J Leukoc Biol 2006; 80(1):1-15.
16. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 2005; 57(1):67-81.
17. Chez MG, Dowling T, Patel PB, Khanna P, Kominsky M. Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. Pediatr Neurol 2007; 36(6):361-5.
18. Li X, Chauhan A, Sheikh AM, et al. Elevated immune response in the brain of autistic patients. J Neuroimmunol 2009; 207(1-2):111-6.
19. Smith SE, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 2007; 27(40):10695-702.
20. Magalhaes ES, Pinto-Mariz F, Bastos-Pinto S, Pontes AT, Prado EA, Deazevedo LC. Immune allergic response in Asperger syndrome. J Neuroimmunol 2009; 216(1-2):108-12.
21. Angelidou A, Alysandratos KD, Asadi S, et al. Brief Report: "Allergic Symptoms" in children with Autism Spectrum Disorders. More than meets the eye? J Autism Dev Disord 2011; 41:1579-85.
22. Jyonouchi H. Autism spectrum disorders and allergy: observation from a pediatric allergy/immunology clinic. Expert Rev Clin Immunol 2010; 6(3):397-411.
23. Theoharides TC. Autism spectrum disorders and mastocytosis. Int J Immunopathol Pharmacol 2009; 22(4):859-65.
24. Theoharides TC, Boucher W, Spear K. Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. Int Arch Allergy Immunol 2002; 128:344-50.
25. Middleton E, Jr., Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol Rev 2000; 52:673-751.
26. Kempuraj D, Madhappan B, Christodoulou S, et al. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. Br J Pharmacol 2005; 145:934-44.
27. Yao Y, Walsh WJ, McGinnis WR, Pratico D. Altered vascular phenotype in autism: correlation with oxidative stress. Arch Neurol 2006; 63(8):1161-4.
28. Jang S, Kelley KW, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK

phosphorylation and activation of AP-1. Proc Natl Acad Sci USA 2008; 105(21):7534-9.
29. Sharma V, Mishra M, Ghosh S, et al. Modulation of interleukin-1beta mediated inflammatory response in human astrocytes by flavonoids: implications in neuroprotection. Brain Res Bull 2007; 73(1-3):55-63.
30. Dirscherl K, Karlstetter M, Ebert S, et al. Luteolin triggers global changes in the microglial transcriptome leading to a unique anti-inflammatory and neuroprotective phenotype. J Neuroinflammation 2010; 7(1):3.
31. Kempuraj D, Tagen M, Iliopoulou BP, et al. Luteolin inhibits myelin basic protein-induced human mast cell activation and mast cell dependent stimulation of Jurkat T cells. Br J Pharmacol 2008; 155:1076-84.
32. Parker-Athill E, Luo D, Bailey A, et al. Flavonoids, a prenatal prophylaxis via targeting JAK2/STAT3 signaling to oppose IL-6/MIA associated autism. J Neuroimmunol 2009; 217(1-2):20-27.
33. Asadi S, Zhang B, Weng Z. et al. Luteolin and thiosalicylate inhibit HgCl(2) and thimerosal-induced VEGF release from human mast cells. Int J Immunopathol Pharmacol 2010; 23(4):1015-20.
34. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. Clin Exp Allergy 2000; 30:501-8.
35. Haleagrahara N, Radhakrishnan A, Lee N, Kumar P. Flavonoid quercetin protects against swimming stress-induced changes in oxidative biomarkers in the hypothalamus of rats. Eur J Pharmacol 2009; 621(1-3):46-52.
36. Kumar A, Goyal R. Quercetin protects against acute immobilization stress-induced behaviors and biochemical alterations in mice. J Med Food 2008; 11(3):469-73.
37. Shimoi K, Okada H, Furugori M, et al. Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. FEBS Lett 1998; 438(3):220-4.
38. Harwood M, nielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC. A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. Food Chem Toxicol 2007; 45(11):2179-205.
39. Kawanishi S, Oikawa S, Murata M. Evaluation for safety of antioxidant chemopreventive agents. Antioxid Redox Signal 2005; 7(11-12):1728-39.
40. De BD, Conti CM, Serroni N, et al. The effect of newer serotonin-noradrenalin antidepressants on cytokine production: a review of the current literature. Int J Immunopathol Pharmacol 2010; 23(2):417-22.