Flavonoids, a prenatal prophylaxis via targeting JAK2/STAT3 signaling to oppose IL-6/MIA associated autism

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1. Introduction

Although the root cause(s) of autism are far from being proven, in recent years, it has been hypothesized that maternal immune activation (MIA) may contribute to the development of autism through chronic induction of pro-inflammatory pathways which start in utero. MIA is used to describe an increase in circulating maternal cytokines in response to an infection during pregnancy (Smith et al., 2007). The ensuing cytokine response and the highly susceptible developmental period in which it occurs may precipitate the neuropathological and behavioral deficits observed in autism and related disorders.

MIA-induced cytokines confer both direct and indirect effects on the fetus. Two methods have been used to study these effects: injecting or up-regulating cytokines during pregnancy in the absence of MIA, or blocking endogenous cytokines or preventing their induction during MIA. A study of the role of TNF-α in LPS-induced fetal loss and growth restriction indicated injection of anti-TNF-α antibodies or an inhibitor of TNF-α synthesis [pentoxifylline] can reduce these effects of LPS. Conversely, injection of TNF-α alone can induce fetal loss (Silver et al., 1994; Xu et al., 2006) which is significantly worse in IL-18 knockout mice, but not in IL-1α/β knockout mice (Wang et al., 2006).

Much of the previous investigations of cytokine mediation of MIA effects on neuropathology and behavior in the offspring have focused on IL-6. This cytokine is involved in the regulation of physiological processes including inflammation and neurodevelopment; making it a particularly appealing candidate molecule for MIA-induced neuro-pathology. Indeed, during neurodevelopment, the signal transducer and activator of transcription-3 (STAT3) pathway, activated by IL-6, maintains homeostasis between neuro- and gliogenesis (He et al., 2005; Murphy et al., 2000).

In support, Samuelsson et al. (2006) injected IL-6 i.p. in pregnant rats for 3 days resulting in severe effects on the offspring. An important finding was that IL-6 mRNA levels remain elevated in the hippocampi of the offspring at 4 and 24 weeks of age; indicative of the ongoing state of immune dysregulation in adult autistic brains. Spatial memory in the water maze, a hippocampal-dependent behavior, was observed in that study. Importantly, the IL-6-treated offspring...
showed that diosmin (10 mg/kg day) was able to block the STAT3
action, suggesting that not only does IL-6 activate the JAK2/STAT3
pathways observed in MIA offspring and potentially autism and
autism-like phenotype (Patterson, 2008).

The effect of flavonoids on JAK/STAT activation has also been
previously described. For example, Park et al. (2008) found that EGCG
inhibits STAT3 activation as an integral part of inhibition of keloid
formation (Park et al., 2008). In an earlier study it was found that sili
binin, a flavonoid, inhibits constitutive activation of Stat3, and causes
caspase activation and apoptotic death of human prostate carcinoma
cells (Agarwal et al., 2007). Prior to this, it was demonstrated that in vivo
treatment of SJL/J mice with quercetin, a flavonoid, (i.p. 50 or 100 μg
every other day) ameliorates experimental autoimmune encephalitis
(EAE) by inhibiting IL-12 production and neural antigen-specific Th1
differentiation. In vitro treatment of activated T cells with this same
flavonoid quaterning blocks IL-12-induced tyrosine phosphorylation of
JAK2, TYK2, STAT3, and STAT4, yielding a reduced IL-12–induced T cell
proliferation and Th1 differentiation (Muthian and Bright, 2004). Our
studies indicated that IL-6 activates the JAK2/STAT3 pathway, as N2a
neuronal cells and brain homogenates from newborn IL-6–induced MIA
(IL-6/MIA) offspring showed increased neuronal JAK2/STAT3 phos-
phorylation. In adulthood, these mice showed deficits in social inter-
action, suggesting that not only does IL-6 activate the JAK2/STAT3
pathway, but that it is also involved in the abnormal behavioral
pathologies observed in MIA offspring and potentially autism and
related disorders. Next we investigated if inhibition of JAK2/STAT3
signaling could attenuate MIA–induced pathologies. Previous research
by our laboratory has shown that bioflavonoids such as epi-gallacea-
techin gallate (EGCG) or luteolin, inhibit IFN–γ–induced STAT1 activation
and attenuate production of pro-inflammatory cytokines in cultured
and primary microglial cells (Giunta et al., 2006; Jagtap et al., 2009;
Rezai-Zadeh et al., 2008).

The goal of the current study was to investigate possible pro-
phylactic effects of two flavonoids which possess potentially better
bioavailability and safety than these previously tested compounds.
These two flavonoids are, luteolin, and its structural analog, diosmin.
We hypothesized that JAK2/STAT3 phosphorylation and signaling as
well as behavioral abnormalities in of IL-6–induced MIA offspring could
be ameliorated with these naturally occurring compounds. Our results
showed that diosmin (10 mg/kg day) was able to block the STAT3
signal pathway; significantly opposing IL-6–induced abnormal behav-
ior and neuropathological abnormalities in MIA/adult offspring. Using
guidelines put forth by the Food and Drug Administration (Reagan-
Shaw et al., 2008), this 10 mg/kg day dose in mice is equivalent to
0.81 mg/kg/day in humans which translates into 48.6 mg/day for a
60 kg person.

2. Methods

2.1. Reagents

Luteolin (>95% purity by HPLC) was purchased from Sigma (St
Louis, MO, USA). Diosmin (>90% purity by HPLC) was purchased from
Axxora (San Diego, CA, USA). Antibodies against JAK2, phospho-JAK2,
STAT3 and phospho-STAT3 were obtained from Cell Signaling Technol-
ogy (Danvers, MA, USA). ELISA kits for tumor necrosis factor-α (TNF–α)
and Interleukin-1β (IL-1β) were obtained from R&D Systems (Minnea-
polis, MN, USA). BCA protein assay kit was purchased from Pierce
Biotecnology (Rockford, IL, USA). Murine recombinant IL-6 was
purchased from ebioscience (San Diego, CA, USA).

2.2. Primary cell culture

Cerebral cortices were isolated from C57BL/6 mouse embryos,
between 15 and 17 days in utero. After 15 min of incubation in trypsin
(0.25%) at 37 °C, individual cortices were mechanically dissociated. Cells
were collected after centrifugation at 1200 rpm, resuspended in DMEM
supplemented with 10% fetal calf serum, 10% horse serum, uridine
(33.6 g/mL; Sigma) and fluorideoxuridine (13.6 g/mL; Sigma),
and plated in 24 well collagen coated culture plates at a density of 2.5 × 10^5
cells per well.

N2a (murine neuroblastoma) cells, purchased from the American
Type Culture Collection (ATCC, Manassas, VA) were grown in com-
plete EMEM supplemented with 1% fetal calf serum. Cells were
plated in 24 well collagen coating culture plates at a density of 1 × 10^5
cells per well. After overnight incubation, N2a cells were incubated
in neurobasal media supplemented with 3 mM dibutryl cAMP in
preparation for treatment.

Cells were treated with 50 ng/mL murine recombinant IL-6 for a
range of time points (0, 15, 30, 45, 60 or 75 min) in the presence or
absence of various concentrations of luteolin (0, 1.25, 2.5, 5, 10,
20 μM) for 30 min.

2.3. Mice

Pregnant C57BL/6 mice, embryonic day 2 (E2) were obtained from
Jackson Laboratory (Bar Harbor, MA) and individually housed and
maintained in an animal facility of the University of South Florida
(USF). All subsequent experiments were performed in compliance
with protocols approved by the USF Institutional Animal Care and Use
Committee. At E12.5, mice were intraperitoneally (i.p.) challenged
(one time only) with murine recombinant IL-6 (5 μg dissolved in
200 μL of PBS/mouse) in the presence or absence of the STAT3 inhibitor
S31–201 (4 μg dissolved in 200 μL of PBS/mouse) and/or treated with
diosmin, administered orally (10 mg/kg/day, 0.005% in NIH31 chow).
Intraperitoneal PBS injection (200 μL) was used as the control for IL-6
administration.

2.4. Western blot

Cultured cells were lysed in ice-cold lysis buffer (20 mM Tris, pH
7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100,
2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4,
1 μg/mL leupeptin, 1 mM PMSF) as described previously (Tan
et al., 2002). Mouse brains were isolated under sterile conditions on
ice and placed in ice-cold lysis buffer. Brains were then sonicated on
ice for approximately 3 min, allowed to stand for 15 min at 4 °C, then
centrifuged at 15,000 rpm for 15 min at 4 °C. Total protein content
was estimated using the BCA protein assay (Pierce Biotechnology) and
 aliquots corresponding to 100 μg of total protein were electrophore-
tically separated using 10% Tris gels. Electrophoresed proteins were
then transferred to nitrocellulose membranes (Bio-Rad, Richmond,
CA), washed in Tris buffered saline with 0.1% Tween-20 (TBS/T), and
blocked for 1 h at ambient temperature in TBS/T containing 5% (w/v)
non-fat dry milk. After blocking, membranes were hybridized over-
night at 4 °C with various primary antibodies. Membranes were then
washed ×3 for 5 min each in TBS/T and incubated for 1 h at ambient
temperature with the appropriate HRP-conjugated secondary anti-
body (1:1000, Pierce Biotechnology). Primary antibodies were diluted
in TBS/T containing 5% BSA and secondary antibodies in TBS containing 5% (w/v) of non-fat dry milk. Blots were developed using the luminol reagent (Pierce Biotechnology). Densitometric analysis was conducted using a FluorO MultiImager with Quantity One™ software (Bio-Rad, Hercules, CA). For phospho-STAT3 detection, membranes were probed with a phospho-Ser727 STAT3 antibody (1:1000) and stripped with stripping solution and then re-probed with antibody that recognizes total STAT3 (1:1000). Similarly for phospho-JAK2 detection, membranes were probed with phospho-JAK2 (1:1000) and stripped and re-probed for total JAK2 (1:1000).

2.5. ELISA cytokine analysis

Mouse brain homogenates were prepared as described above and used at a dilution of 1:10 in PBS for these assays. Brain tissue-solubilized cytokines were quantified using commercially available ELISAs that allow for detection of IL-1β, and TNF-α. Cytokine detection was carried out according to the manufacturer’s instruction. Total protein content was determined as described above and data represented as pg of cytokine/mg total cellular protein for each cytokine.

2.6. Behavioral testing

Open field (OF) — The OF behavioral analysis is a test of both locomotor activity and emotionality in rodents (Radyushkin et al., 2009). Mice were placed in a 50 × 50 cm white Plexiglas box brightly lit by fluorescent room lighting and six 60 W incandescent bulbs approximately 1.5 m above the box. Activity was recorded by a ceiling-mounted video camera and analyzed from digital video files either by the automated tracking capabilities of Ethovision or counted using the behavior tracker (version 1.5, www.behaviortracker.com), a software-based event-recorder. The total distance moved and numbers of entries into the center of the arena (central 17 cm square) were determined in a 10 minute session.

Social interaction (SI) — The testing apparatus consisted of a 60 × 40 cm Plexiglas box divided into three chambers. Mice were able to move between chambers through a small opening (6 × 6 cm) in the dividers. Plastic cylinders in each of the two side chambers contained the probe mice, and numerous 1 cm holes in the cylinders enabled test and probe mice to contact each other. Test mice were placed in the center chamber, with an overhead camera recording their movements. Mice were allowed 5 min of exploration time in the box, after which an unfamiliar, same-sex probe mouse from the same experimental group was placed in one of two restraining cylinders (Radyushkin et al., 2009). The Ethovision software (Noldus, Leesburg, VA) program measured time spent in each of the three chambers, and social preference was determined as follows: (% time spent in the social chamber) − (% time spent in the opposite chamber).

2.7. Statistical analysis

All data were normally distributed; therefore, in instances of single mean comparisons, Levene’s test for equality of variances followed by t-test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by post hoc comparison using Bonferroni’s method. Alpha levels were set at 0.05 for all analyses. The STATistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, IL, USA) was used for all data analysis.

3. Results

3.1. Luteolin inhibits IL-6 induced neuronal JAK2/STAT3 phosphorylation

To confirm the role of IL-6 in regulating the JAK2/STAT3 pathway, we treated murine neuron-like (N2a) cells and primary cultured neuronal cells with 50 ng/mL of murine recombinant IL-6 in a time-dependent manner. Western blot analysis of cell lysates showed that IL-6 treatment leads to a time-dependent increase in JAK2 (Fig. 1A) and STAT3 (Fig. 1C) phosphorylation. Densitometric analysis indicated significant and steady increases in JAK2 phosphorylation (**P < 0.005) beginning at 30 min and continuing until 75 min when the timed analysis was concluded (Fig. 1A). Densitometric analysis of STAT3 phosphorylation (**P < 0.005) showed a significant and maximal increase at 30 min, with no further significant increases at subsequent time points.

We next examined the effects of luteolin on IL-6 induced JAK2/STAT3 phosphorylation. Murine N2a cells and primary cultured neuronal cells were again challenged with 50 ng/mL murine recombinant IL-6 and co-treated with increasing concentrations of luteolin (0–20 μM) for 30 min. Following Western blot analysis of cell lysates, we found that luteolin reduces IL-6 induced JAK2 phosphorylation (Fig. 1B) and STAT3 phosphorylation (Ser727) (Fig. 1D) in both murine N2a and primary neurons in a dose dependent manner with significant reductions beginning at 10 μM. Densitometric analysis showed that luteolin inhibited JAK2 and STAT3 (Ser727) phosphorylation by almost 50% (**P < 0.01 and **P < 0.001, respectively). It is important to note that luteolin did not affect apoptosis levels of JAK2 or STAT3.

3.2. STAT3 inhibitor, S31-201, and diosmin reduce JAK2/STAT3 phosphorylation and pro-inflammatory cytokine production in the brain tissues of IL-6/MIA newborn mice

In this study, we sought to extend the in vitro results to an animal model of MIA-induced autism by examining the effects of STAT3 inhibitor (S31-201) and diosmin (a flavonoid structural analog of luteolin), on JAK2/STAT3 phosphorylation and signaling. When either agent was co-administered to pregnant mice intraperitoneally with IL-6, JAK2, STAT3 phosphorylation and pro-inflammatory cytokine levels were both significantly reduced in the brain homogenates of newborn mice. Western blot analysis of brain homogenates shows that both S31-201 and diosmin significantly reduce IL-6 induced JAK2 (Fig. 2A) and STAT3 (Fig. 2B) phosphorylation (**P < 0.005).

Pro-inflammatory cytokine ELISA showed significant increases in TNF-α and IL-1β levels in the brain homogenates of newborn mice from IL-6 treated dams compared to those of control dams (Fig. 3). These increases were significantly reduced by almost 50% in the presence of S31-201 or diosmin (**P < 0.01) (Fig. 3).

3.3. Maternally blocking the STAT3 signal pathway with the STAT3 inhibitor, S31-201, and diosmin opposes IL-6-induced abnormal behavior in MIA/adult offspring

This experiment was aimed at determining whether diosmin would attenuate behavioral abnormalities observed in the adult offspring of IL-6 treated dams. Pregnant mice were treated one time with 5 μg/mL IL-6 in the presence or absence of S31-201 (4 μg/mL) or diosmin (10 mg/kg/day diosmin) administered orally in chow. We also treated control mice (non-IL-6 treated) with the STAT3 inhibitor, S31. The adult offspring of these mice were examined for behavioral outcomes using the open field and social interaction tests to examine anxiety and social interaction, respectively. Our results demonstrate that S31-201 or diosmin co-treatment significantly attenuates the behavioral deficits seen in the adult offspring of IL-6 treated animals. In the open-field test, offspring of mice treated with either S31-201 or diosmin showed behaviors comparable to that of control mice, entering the center more often than IL-6 treated animals (**P < 0.01) (Fig. 4A). In addition, an ANOVA on time spent in the inner section showed a significant main effect of group (P < 0.005) and LSD post hoc tests showed that the IL-6/MIA mice spend less time in the inner section compared to S31-201 or diosmin treated mice (P < 0.05). As these could be due to simply increased locomotion in one group,
ANOVA on distance traveled in the inner section was performed and did not indicate a main effect (\(P = 0.069\)). Analyses of distance traveled in the outer section did not reveal a significant difference or a statistical trend towards a significant difference (\(P > 0.15\)) among groups. We did not find a significance between PBS-treated mice and S31-treated control (non-IL-6 injected) mice (\(P > 0.05\) with \(n = 5\)).

In the social interaction test, the adult offspring of control mice show a strong preference for the social chamber almost double that of the adult offspring of IL-6 treated mice. To quantify social interaction, exploration demonstrated as sniffing time was analyzed via ANOVA. There was a main effect of group (\(P < 0.05\)); the S31-201 or diosmin mice exhibited an increase in sniffing compared to IL-6/MIA mice (\(P < 0.05\)). We did not observe any social grooming, chasing, dominant mounts, pinning, boxing, or biting. There were no significant differences in total move time (\(P > 0.07\)) and total distance traveled (\(P > 0.05\)).

This social impairment observed in IL-6 adult offspring was significantly attenuated by maternal co-treatment with S31-201 or diosmin as these mice show a preference for the social chamber comparable to that of control adult offspring (\(**P < 0.005\) (Fig. 4B)). Considering the above data, it can be seen that IL-6 induced JAK2/STAT3 phosphorylation plays an essential role in precipitating behavioral abnormalities seen in the adult offspring of IL-6 treated dams and regulation of this pathway by diosmin can attenuate these behavioral abnormalities.

3.4. Diosmin reduces pro-inflammatory cytokines and STAT3 phosphorylation in IL-/MIA adult offspring

After behavioral testing, adult offspring were sacrificed to confirm that inhibition of STAT3 phosphorylation by diosmin attenuates IL-6/JAK2/STAT3 induced behavioral abnormalities. At sacrifice, brain homogenates were prepared from offspring of control mice and mice treated with IL-6, IL-6/S31-201, and IL-6/Diosmin. Pro-inflammatory cytokine ELISA showed significant increase in TNF-\(\alpha\) and IL-1\(\beta\) levels in the homogenates of IL-6 adult offspring. Maternal co-treatment with S31-201 or diosmin significantly reduces TNF-\(\alpha\) and IL-1\(\beta\) levels. 

![Figures A, B, C, D](image-url)
cytokine levels significantly, with diosmin showing slightly more
significant reductions as shown in Fig. 5 (**P < 0.05).

Western blot analysis of phospho- and total STAT3 shows that IL-6
treatment of dams increases STAT3 phosphorylation in the brain
homogenates of adult offspring while co-treatment with either S31-
201 or diosmin leads to a significant reduction of STAT3 phosphor-
ylation (**P < 0.005) (Fig. 6).

4. Discussion

Elucidating the mechanisms and pathways involved in neurode-
velopmental disorders such as autism is important in not only
understanding the etiology of these disorders but also to discover
ey early diagnostic markers, and prophylactic therapies, in addition to
therapeutic strategies to attenuate the associated symptoms. Previous
research by Smith and colleagues has supported the role of exogenous
IL-6 in precipitating the behavioral deficits and increases in pro-
inflammatory cytokine release seen in MIA offspring, and potentially
autistic individuals, in addition to demonstrating that its inhibition
can attenuate these pathologies (Smith et al., 2007). In this study, we
further examined the role of IL-6 in MIA by characterizing the role of JAK2/STAT3 phosphorylation in precipitating behavioral and pathological outcomes. We also sought to determine if inhibition of this phosphorylation had the ability to attenuate these behavioral deficits and/or pathologies observed in the adult offspring of IL-6/MIA mice. Previous research by our laboratory has shown that bioflavonoids regulate STAT1 phosphorylation, as we showed that luteolin (Rezaizadeh et al., 2008) or EGCG (Giunta et al., 2006) inhibited IFN-γ induced STAT1 phosphorylation. We therefore sought to examine if diosmin, a luteolin analog with better bioavailability, had similar effects, inhibiting IL-6 induced STAT3 phosphorylation.

We first aimed to confirm the role of IL-6 induced JAK2/STAT3 phosphorylation in precipitating pathological and behavioral deficits seen previously in IL-6/MIA animal models. The results of our study demonstrate that IL-6 induces JAK2/STAT3 phosphorylation resulting in the release of pro-inflammatory cytokines both in vitro and in vivo. Western blot analyses of cell lysates from murine N2a cells and primary cultured neuronal cells showed time-dependent increases in JAK2/STAT3 phosphorylation following IL-6 treatment. Analysis of brain homogenates of newborn mice from IL-6 treated dams similarly showed increases in JAK2/STAT3 phosphorylation, in addition to increases in released pro-inflammatory cytokines TNF-α and IL-1β.

We measured IL-6 levels in brain homogenates from the offspring using ELISA. The level of IL-6 was undetectable which is in contrast to the results previously reported by Samuelsson et al. (2006). As previously mentioned, this group found that IL-6 mRNA levels remain elevated in the hippocampi of the offspring at 4 and 24 weeks of age; indicative of the ongoing state of immune dysregulation in adult autistic brains. We thus suggest IL-6 itself may not be a contributory factor for the in vivo chronic inflammatory pathogenic affects, but rather for the in utero effects. Therefore measuring IL-6 in the offspring may not be representative of its exposure in utero. Rather, the indirect effects of IL-6 pro-inflammatory activation in utero of the JAK2/STAT3 pathway, as evidenced by increased TNF-α and IL-1β production, may be at play in an autism mouse phenotype. However, further studies will be required to clearly define the role of IL-6 in ongoing autism. Thus, with evidence that JAK2/STAT3 phosphorylation induced pathological outcomes in the offspring of IL-6 treated animals, we next sought to examine whether or not this phosphorylation contributed to the behavioral deficits observed in the offspring of IL-6 treated dams.

There is much epidemiologic evidence indicating that environmental contributions, including prenatal infections which can lead to MIA, may lead to the genesis of autism (Arndt et al., 2005; Libbey et al., 2005). Previous animal models, developed by Fatemi and Folsom (2009) and others, have shown that immune challenges during pregnancy lead to abnormal brain structure and function in the exposed offspring that replicate abnormalities observed in brains of subjects with autism (Fatemi et al., 2005, 2008; Meyer et al., 2006, 2007; Shi et al., 2003). Abnormal CNS changes in the offspring following infection at E9, which corresponds to infection at the middle of the first trimester (Fatemi et al., 2005; Shi et al., 2003), and E18, which corresponds to infection in late second trimester (Fatemi et al., 2008) have been previously shown. Importantly E16 immediately follows the period of neurogenesis of hippocampal pyramidal cells (E11–E15.5) (Rodier, 1980). Thus we hypothesized that middle second trimester infection (E12.5, E15 and E17) in mice would alter brain cytokine expression of the offspring since Fatemi and Folsom (2009)
found that infection at E16 leads to altered expression of many brain genes in the hippocampi of the exposed mouse offspring.

Our results showed that the adult offspring of IL-6 treated animals displayed behavioral deficits in social interaction and regulation of anxiety that are reminiscent of autism. We also found neuropathology previously described (Turner, 1999) including increase pro-inflammatory cytokine release and increased STAT3 phosphorylation. In many previous works by other groups the pre-pulse inhibition (PPI) test is particularly informative for animal models of autism, because there are existing models for understanding the neural circuitry of startle gating; a process known to involve inhibitory cortico-striatal neural circuits (Braff et al., 2001). Furthermore, sensorimotor gating deficits have been reported in a family of neurodevelopmental disorders and are not specific to autism. Whereas Ornit et al. (1993) reported equivocal PPI results in autistic children, others reported PPI deficits in adults with Asperger syndrome (McAlonan et al., 2002), children with Tourette syndrome (Swerdlow et al., 2001), and men with fragile X syndrome (Frankland et al., 2004). To date, only one published study on adults with autism (14 adult men diagnosed with autism and 16 typically developing normal comparison (NC) participants) has been conducted on the subject and it concluded that PPI deficits may only be indirectly linked to one of the hallmark features of autism. With this in mind we did not measure PPI in this study because if it were normal, it would have no scientific impact on the validity of the model, and if it were abnormal, it in itself would not be a measure of true neuropsychiatric impairment, rather the functional behavioral tests we performed would.

As a confirmation of our results, we examined the effects of luteolin and diosmin in regulating IL-6 induced JAK2/STAT3 phosphorylation in vitro and if this could attenuate the pathologies and behavioral deficits previously described. When murine N2A cells and primary cultured neuronal cells were co-treated with IL-6 and luteolin, we observe a concentration dependent decrease in JAK2/STAT3 phosphorylation, as evidenced by Western blot analysis of cell lysates. We next examined the in vivo effects of diosmin, with the STAT3 inhibitor S31-201 as a positive control. When pregnant mice were co-treated with IL-6 and either diosmin, or S31-201, we observed an attenuation of the behavioral deficits in the adult offspring of diosmin and S31-201 co-treated animals as they showed social behaviors comparable to that of control mice. Furthermore, when brain homogenates of these adult offspring were examined, we saw decreased STAT3 phosphorylation with decreased pro-inflammatory cytokine secretion.

Taken together, our results show that IL-6 induced JAK2/STAT3 phosphorylation plays an integral role in the execution of IL-6/MIA mediated pathological effects. Indeed, inhibition of this phosphorylation was able to attenuate both behavioral deficits and pathological outcomes such as increased inflammation. In the future, we will further examine whether the JAK2/STAT3 signal pathway activation is specifically involved in brain histological abnormalities in IL-6-induced MIA (presumably resulting in development of abnormal behavior in adult offspring that mimics features of autism). In addition, we intend to fully characterize the mechanisms by which diosmin regulates JAK2/STAT3 phosphorylation in addition to qualifying its potential effect on improving abnormal, autistic like social behaviors in IL-6/MIA offspring in adulthood via its attenuation of the JAK2/STAT3 signal pathway. These studies could lay the foundation for autism clinical trials with diosmin diet supplementation in the future.

This may be not only effective, but safe. Indeed diosmin is a natural flavonoid isolated from various plant sources or derived from the flavonoid hesperidin. First used as a therapy in 1969, diosmin is currently considered a vascular-protecting agent and has been used for treatment of chronic venous insufficiency/varicose veins (Jantet, 2002), hemorrhoids (Jantet, 2000), lymphedema (Pecking et al., 1997), and diabetes (Lacombe et al., 1989). The compound also exhibits anti-inflammatory, antioxidant, and antimutagenic properties (Kuntz et al., 1999). Furthermore, marketed formulations 90% diosmin, 10% hesperiden pose little to no side effects. Taken together, there is convincing evidence from preliminary studies regarding efficacy, as well as published studies regarding safety in humans, that diosmin is a safe and potentially efficacious treatment for autism. Furthermore, the dose found to be effective in this animal model (10 mg/kg/day) translates into a human dose of approximately 48.6 mg/day for a 60 kg person. While not reasonably achievable through consumption of foods containing diosmin, this concentration may be provided through a daily oral supplement. Therefore the data from this study will be used to help determine the shape of the dose response, to begin to achieve the optimal dosing schedule for future human clinical trials.

Acknowledgments

This work was supported by the Silver Foundation. J.T. holds the Sliver Chair in Developmental Neurobiology. We thank Drs. Yuyan Zhu, Huayan Hou and Kavon Rezai-Zadeh for helpful advice.

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