The Effects of Chrysin, a *Passiflora incarnata* Extract, on Natural Killer Cell Activity in Male Sprague-Dawley Rats Undergoing Abdominal Surgery

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Chrysin, a passion flower extract, may be beneficial because of its potential to attenuate surgical suppression of natural killer (NK) cell activity. We divided 37 male Sprague-Dawley rats into 3 treatment groups: (1) rats undergoing abdominal surgery and administered isoflurane and a 5% solution of dimethyl sulfoxide in saline (vehicle), (2) rats undergoing abdominal surgery and administered isoflurane and chrysin solubilized in 5% dimethyl sulfoxide, and (3) rats not undergoing surgery but administered isoflurane and chrysin. Natural killer cell activity was measured before and 24 hours after the experiment.

*Analysis of covariance, with preoperative NK cell activity as the covariate, was used to compare differences in NK cell activity among groups. The Scheffe procedure was used to make post hoc comparisons. Analysis revealed a significant difference (*P = .006*) such that group 2 had significantly less NK cell suppression compared with groups 1 and 3. These findings suggest that chrysin may attenuate surgical suppression of NK cell activity, thereby minimizing metastatic spread of cancer.*

Keywords: Anxiety, chrysin, natural killer cells, passion flower.

The American Cancer Society estimates that nearly 600,000 Americans will die of cancer in 2006.[1] Although treatment options for solid tumor cancers frequently include surgery, it has been known since 1984 that surgery is associated with suppression of natural killer (NK) cell activity and, thereby, may contribute to cancer cell metastasis.[2] This creates a paradox. Patients with solid tumor carcinomas require surgery; however, surgery suppresses antimetastatic immunity, thereby contributing to cancer cell metastasis. Accompanying this life-changing diagnosis and subsequent surgery is an increase in anxiety levels. Without effective coping measures, patients may experience sustained anxiety leading to initiation of the stress response. Prolonged activation of the stress response may lead to a decrease in the immune response and place patients at increased risk for secondary illnesses or spread of cancer cells.[3,4] Activation of the hypothalamic-pituitary-adrenal axis is considered the key mechanism of brain-mediated and stress-induced modulation of immunity and related disease processes. Activation of the hypothalamic-pituitary-adrenal axis may initiate suppression of NK cell activity by an actual decrease in the numbers of NK cells available or a decrease in function.[5] Research suggests that controlling the stress response may have an essential role in inhibiting stress-induced suppression of NK cell activity.[6]

Natural killer cells are a subpopulation of lymphocytes that spontaneously recognize and kill virally infected cells and a variety of tumor cells during metastasis. Mature NK cells are exported to the periphery and are found in the blood, all of the lymphoid organs, and some parenchymal tissues such as the lungs and liver. Natural killer cells have a heterogeneous arsenal of surface receptors that allow them to respond to cytokines, stress signals, and inducible molecules that are expressed after target-cell transformation, such as occurs with the coating of target cells with immunoglobulin G. Thus, NK cells are crucial for defense against infectious diseases and cancer.[7]

Several factors have been shown to affect NK cells activity, including stress, anxiety, surgical procedures, and certain anesthetics.[8-10] Since the characterization of NK cells in the 1970s, it has become apparent that emotional and physical stress can suppress NK cell activity.[11] Hence, surgical suppression of NK cell activity accompanied by a prolonged stress response may magnify postoperative NK cell suppression and place patients at risk for metastatic

spread of cancer. Koga and colleagues[12] found that NK cell activity was significantly lower in patients with anxiety about their cancer compared with those without the same level of anxiety. Because NK cells are important in immunosurveillance against tumor development, adequate management of pain and anxiety may benefit patients.

An exhaustive line of research in rodents and humans clearly shows that NK cell activity is suppressed during stressful events such as surgical intervention, thus promoting metastatic spread of cancer.[13,14] Therefore, control of anxiety must be an essential element of care. This is often accomplished through the use of pharmaceutical agents. Benzodiazepines have been shown to reduce stress levels during surgical intervention. However, long-term use of benzodiazepines is associated with tolerance and dependency leading to the need for exploration of alternative means of producing anxiolysis. Chrysin, an extract of passion flower species (*Passiflora*) has demonstrated anxiolytic properties similar to benzodiazepines but may not have the associated side effects.[15-18]

Passiflora incarnata, used as a sleep aid or an anxiolytic, is available through several herbal websites and health stores in the United States. The aerial parts of the plant were used for their anxiolytic and tranquilizing properties in the mid 16th century in the Peruvian Andes by the Spanish and later along the American Gulf coast by Native Americans. The chemical composition of *P incarnata* includes maltol, flavonoids (orientin, isoorientin, vitexin, and isovitexin), and indole alkaloids (harman, harmin, harmalin, harmol, and harmalol). Only the aerial components of maltol and flavonoids are used for their anxiolytic and tranquilizing effects; the indole alkaloids are thought to be stimulants.[19,20] The anxiolytic effect of flavonoids such as chrysin is blocked by the administration of flumazenil, suggesting that chrysin binds to the γ-aminobutyric acid (GABA) receptor, possibly the α subunit.[21]

The purpose of this study was to examine the efficacy of administration of chrysin to male Sprague-Dawley rats 30 minutes before abdominal surgery in attenuating surgical suppression of NK cell activity postoperatively.

Materials and Methods

The study was approved by the Wilford Hall Medical Center Institutional Animal Care and Use Committee. Mature male Sprague-Dawley rats, weighing 225 to 250 g, were acclimatized to the vivarium and handled for 14 days. Two days before surgery, all rodents in all groups had whole blood drawn via an intracardiac puncture under brief isoflurane anesthesia to determine basal NK cell activity. On the day of surgery, animals were administered chrysin (Sigma-Aldrich, Inc, St Louis, Missouri), 3 mg/kg, or the vehicle via intraperitoneal injection 30 minutes before abdominal surgery. The dose and timing of the chrysin administration were derived from the work of Wolfman et al[17] and Zanoli et al.[20] All animals were anesthetized with 5% isoflurane anesthesia and maintained with 2.5% isoflurane. The following were the 3 treatment groups: (1) rats undergoing abdominal surgery and administered isoflurane and a 5% solution of dimethyl sulfoxide in saline (vehicle), (2) rats undergoing abdominal surgery and administered isoflurane and chrysin solubilized in 5% dimethyl sulfoxide, and (3) rats not undergoing surgery but administered isoflurane and chrysin. Surgical animals were shaved, and a standard laparotomy was performed. This standard laparotomy has been used extensively to induce an equivalent magnitude of surgical stress in rats.[22] Surgery consisted of a 4-cm midline incision through the skin and abdominal muscle wall, followed by the externalization of a 10-cm segment of the small intestine. The intestine was gently rubbed between 2 pieces of gauze in 4 locations as a standard irritant to promote the release of local inflammatory factors. After 4 minutes, the intestines were returned to the abdominal cavity, and the muscle and skin layers were sutured. The rats were recovered from anesthesia and returned to their cages for 24 hours. All animals received postoperative analgesia by subcutaneous injection of buprenorphine. At 24 hours postoperatively, blood was drawn from all animals via an intracardiac puncture under brief isoflurane anesthesia for a postexperimental NK cell assay.

The NK cell activity assays were performed by the laboratories of the Wilford Hall Medical Center, 59th Research Squadron, Lackland Air Force Base, TX, per the chromium release assay, which has been the "gold" standard since 1968.[23] Briefly, YAC-1 cells, a standard cell line for the assessment of rodent NK cell activity, were incubated with chromium 51 to label them with a radioactive substance for measurement later. The irradiated target cells were serially diluted to achieve various effector (NK) to target (YAC-1) ratios. Target cells and NK cells were incubated together for 4 hours so that NK cells would have time to "kill" the irradiated YAC-1 cells. The supernatant was harvested to determine chromium 51 emission using a Beckman Liquid scintillation counter. (Beckman Coulter, Inc, Fullerton, California) Lytic unit activity, the ability of the NK cells to kill the target YAC-1 cells, was calculated by using the standard method of lytic calculations.[24] The numbers of NK cells were measured by flow cytometry.

Results

The determination of effect size was based on previous work by Benschop et al.[25] By using the data in this study and the Cohen formula[26] (Cohen $d = M_1 - M_2/\sigma$ pooled, where σ pooled $= \sqrt{[(\sigma_1^2 + \sigma_2^2) / 2]}$), we calculated that a large effect size of 0.67 would be appropriate. Hence, using an effect size of 0.067, a power of 0.80, and an α of .05, we used G Power 2.1.2 and calculated that a total

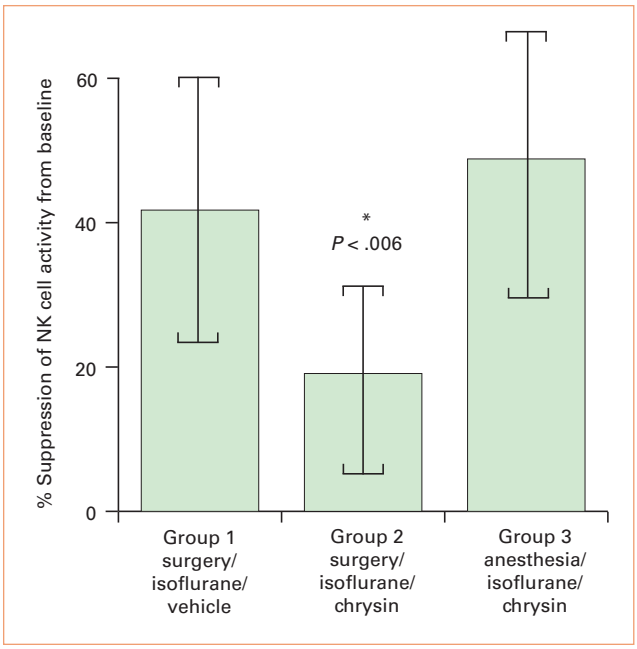

Figure 1. Percentage Suppression of Natural Killer (NK) Cell Activity Compared With Baseline Among Groups

Bars show the mean and errors bars the 95% confidence interval of the mean.

* Indicates a significant difference in the percentage of change in NK cell activity from baseline.

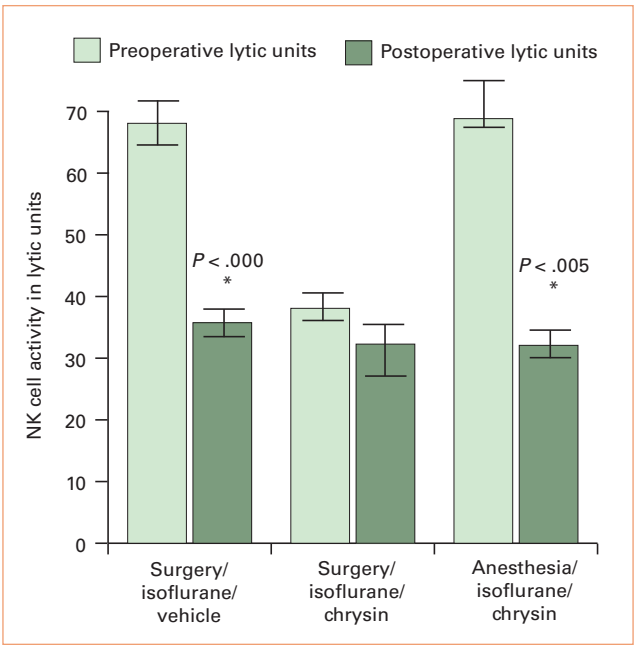

Figure 2. Comparison of Preexperimental and Postexperimental Natural Killer (NK) Cell Activity Among the Groups

Bars show the mean and error bars the mean ± 1 SE.

* Indicates a significant difference in NK cell activity measured in lytic units.

sample size of 33 rats (11 per group) was needed. Four additional animals were used for model development, for a total of 37 animals.

Analysis of covariance with the preoperative NK cell activity as the covariate was used to compare NK cell activity among groups. The Scheffe procedure, a conservative post hoc test, was used to make comparisons between groups. Analysis revealed a significant difference ($F = 11.99$; $P = .006$) such that group 2 had significantly less NK cell suppression compared with groups 1 and 3. Because there was no significant difference between groups with regard to baseline NK cell activity ($F = 3.76$; $P = .06$), we chose to represent the data as percentage of change in baseline. Figure 1 represents the data in clinically relevant terms, using the percentage of suppression in NK cell activity among the groups. Group 2 had a 19% decrement in lytic activity compared with preexperimental (baseline) values, whereas the other 2 groups had more than 40% suppression of NK cell activity.

Figure 2 graphically represents a comparison of preexperimental NK cell activity in lytic units with postexperimental NK cell activity among the groups. As expected, all groups showed a decrease in NK cell activity compared with baseline preexperimental values. However, whereas postexperimental NK cell activity was significantly suppressed compared with preexperimental values in groups 1 and 3, the group 2 rats did not have significant suppression of NK cell activity ($P = .006$). Furthermore, NK cell numbers were not significantly different among the groups ($F = 3.07$; $P = .9$), suggesting that changes in NK cell activity were due to lytic activity only (Figure 2).

Discussion

To our knowledge, this study is the first to examine the effects of chrysin, a *Passiflora* extract, on NK cell activity. Our finding that chrysin, a purported GABA receptor agonist, inhibits surgical suppression of NK cell activity is congruent with findings that suggest other GABA agonists such as the benzodiazepines enhance the immune system in rodents that have undergone surgical stress. For example, an abdominal laparotomy with intestinal manipulation, similar to the technique used in our study, produced immunosuppression in rodents, specifically a decrease in thymus, spleen, and peripheral lymphocyte cell numbers. The benzodiazepines midazolam and alprazolam in doses equivalent to clinically relevant human doses (1 mg/kg each), significantly attenuated the surgical immunosuppression produced by the laparotomy.[27,28] Moreover, several lines of research suggest that benzodiazepines effectively inhibit stress-related suppression of NK cell activity and enhance NK cell function. Rodent studies in which mice were subjected to handling stress or injected with stress hormones have shown that diazepam (2-5 mg/kg) and alprazolam (0.5-1 mg/kg) have immunoenhancing activity as evidenced by preservation

of NK cell activity after handling stress and after chemically induced stress via treatment with corticotropin releasing hormone.[29-31]

Our findings are also consistent with studies suggesting that other herbal preparations are effective in preserving NK cell activity. A 10-day course of Ganopoly, an herbal extract from the Ganoderma lucidum plant, which is used extensively in Malaysian traditional medicine to treat cancer and hepatic disease, was found to significantly increase cytotoxic T-lymphocyte and NK cell activity. Furthermore, in mice with cancer, tumor size was significantly reduced in a dose-dependent manner.[32] Similarly, the administration of Juzen-taiho-to significantly increased NK cell number and function compared with untreated mice. More important, Juzen-taiho-to prolonged the survival of mice injected with leukemic cells compared with mice that were not administered Juzen-taiho-to.[33] Taken together, the findings from these studies and ours suggest that continued research is warranted regarding the possible benefit of herbal preparations or extracts in the treatment of cancer.

The interesting finding that chrysin may, in fact, suppress NK cell activity under nonsurgical conditions is also supported by other research. A series of studies have shown that adult male Wistar rats and Swiss albino mice have suppressed humoral (NK cell–type response) and cellular (B and T cell–type response) immune responses when administered diazepam (2.5 or 5 mg/kg) or alprazolam (0.5 or 1 mg/kg) under nonstressful conditions. However, diazepam and alprazolam attenuate the stress-induced suppression of immune function when administered before a stressful event. The authors suggested that these contradictory findings were mediated via central GABA (benzodiazepine) receptors rather than by peripheral benzodiazepine receptors.[34,35] Similar to these findings, our study showed that the administration of chrysin to rats in group 3, the nonstressed rats, produced suppression of NK cell activity, whereas administration of chrysin to the stressed rats in group 2 attenuated the surgical suppression of NK cell activity.

Research in the 1990s suggested that benzodiazepines produced positive immunoenhancing and antimetastatic effects through preservation of NK cell activity. The renewed interest in peripheral benzodiazepine receptors has, through advanced molecular techniques, demonstrated more clearly the interactions among the immune system, cancer, and benzodiazepine receptors.[36] Specific peripheral benzodiazepine receptor ligands have been described and have been suggested to be effective anticancer compounds against hepatic cancer and leukemia by promoting cancer cell apoptosis (cell death) in in vitro and in vivo experiments.[37-39] Furthermore, chrysin and other natural flavones have emerged in recent studies as potential chemotherapeutic agents.[40,41] Findings from in vitro studies suggest that one way chrysin may be effective as an anticancer agent is through induction of apoptosis in cancer cells.[42,43]

Several studies of humans with various cancers have suggested that patients with higher NK cell activity have a longer metastasis-free survival time compared with patients with lower NK cell activity. Prognosis for survival has been positively correlated with normal NK cell activity in studies of colorectal cancer, prostate cancer, breast cancer, and carcinoma of the lung.[44-47] Whether low NK cell activity promotes cancer metastasis or is the result of cancer has not been elucidated. We found that the preoperative administration of chrysin inhibited surgical suppression of NK cell activity in rats that underwent an abdominal laparotomy using isoflurane anesthesia compared with rats that underwent surgery using isoflurane anesthesia and were administered vehicle. We also found that rats administered chrysin and isoflurane that did not undergo surgery had significantly suppressed NK cell activity compared with rats administered chrysin and isoflurane that did undergo surgery. Further studies are required to fully elucidate the effects of chrysin on NK cell activity under surgical and nonsurgical conditions.

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