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EFFECTS OF DIET ON IMMUNE CELLS WITHIN THE CENTRAL NERVOUS SYSTEM

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Introduction

Chronic inflammation is a critical component of many health conditions including both chronic pain and obesity. Obese individuals are at greater risk for developing chronic pain (1–3) and the persistent inflammation associated with obesity may be a contributing cause of concurrent pain (4, 5). Obesity is characterized by an increase in adiposity that is typically due to long-term energy imbalance resulting from excess energy intake and insufficient energy expenditure. With obesity, white adipose tissue undergoes numerous changes that lead to the recruitment of immune cells, such as macrophages, and the release of pro-inflammatory factors. Additionally, the increased caloric intake that characterizes obesity is usually a result of a poor-quality diet. Many components of a poor diet can independently activate the immune system (6). Therefore, obese individuals who consume a poor diet may be at greater risk for chronic pain through increased immune system activation.

Previously, we investigated the impact of diet composition on pain and recovery in rodents. To address this question, our group developed a Standard American Diet (SAD) with similar amounts of carbohydrates, fats, and protein to a typical human American diet (7). SAD consumption led to more pronounced hypersensitivity, prolonged recovery, and microglial/macrophage activation in both rats and mice (7, 8).

To investigate whether these poor outcomes could be reversed, we developed an antiinflammatory diet (AID) using ingredients that have been shown to limit inflammation. Using the AID either prophylactically or as an intervention decreased recovery times in mice (8), providing support for the use of dietary interventions to alleviate pain. Previous investigations in humans have used specific diets in order to reduce pain (9–11).

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For example, a Mediterranean diet has been shown to reduce pain in adults with rheumatoid arthritis (10–12). Another study examining the effect of diet on osteoarthritis pain found that 18 months of dietary intervention involving low-fat, high vegetable meals and meal replacement shakes reduced pain and improved function in the knee (13). Therefore, our results in rodent models are in line with those found in human pain patients.

We demonstrated previously that the SAD led to systemic inflammation, evidenced by an increase in pro-inflammatory cytokines (7). However, there is an established role for the central nervous system in mediating pain responses. Multiple studies have revealed the importance of microglia in the development and maintenance of chronic pain (14–18). Immunohistochemical analysis demonstrated that our SAD led to an increase in microglia/macrophages within the spinal dorsal horn of rats (7). This is consistent with other reports in which high-fat diet-induced obesity leads to microglial activation in the brain (19–21).

A clear sex difference has been uncovered in pain maintenance mechanisms, in which males tend to utilize microglia, whereas females utilize T cells (22), though this may change during pregnancy (23). Furthermore, there is evidence that T cells infiltrate the spinal cord following a nerve injury and contribute to mechanical sensitivity (24). It has also been shown that high-fat diet-induced obesity leads to recruitment of immune cells into the central nervous system (25). Together, these studies illustrate that the immune system is involved in pain from injury and that poor diet can activate the immune system in the periphery and central nervous system.

In the current study, we sought to define the effects of our SAD on T cell infiltration into the brain and spinal cord at different diet-exposure times. Additionally, we wished to gauge the potential of the AID to prevent or reverse the immune cell infiltration into the central nervous system following weeks of SAD consumption. To accomplish this, we used flow cytometry to assess immune cell infiltration into the central nervous system following a postoperative pain model in male and female mice.

Materials and Methods

Experimental Design

During 15 weeks of diet exposure, all mice were weighed weekly. After 15 weeks, an injury was induced using a paw incision model in all animals (26). At the time of injury, a subset of animals from the SAD group were switched to the AID group. Animals were sacrificed at day 3 and week 15 of SAD consumption, as well as day 3 and 7 post-paw incision (Figure 1).

Animals

Male and female (n = 10/group/time, 6 weeks of age at onset) CD1 mice (ICR:CrI) were housed in groups of 2–4, under a 12 h light cycle (lights on at 07:00 h) and provided with standard chow (REG; NIH-31, Harlan Teklad) and sterile water. All mice were fed standard chow for two weeks before introduction to the experimental diets. Mice were assigned to either *ad libitum* regular chow (REG, n = 40, 20 males) or a Standard American Diet (7, 8) (SAD, n = 58, 29 males). At week 15, a subset of mice were switched from the SAD and

onto the AID (n = 20, 10 males). All animals used in the present study were obtained, housed, cared for and used in accordance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

Diet Compositions

The SAD was developed by our group and used previously (7). It contains high amounts of carbohydrates, fats, and omega-6 fatty acids with Primex as a source of trans fats. The AID was also developed by our group and was recently used in mice (8). The AID contains translatable amounts of epigallocatechin gallate (EGCG), sulforaphane, resveratrol, curcumin, and ginseng. Both diets might be considered high-fat diets but the main sources of fat in the SAD are from lard, beef tallow, milkfat and hydrogenated vegetable shortening, whereas the main sources in the AID are from flaxseed and soybean oil. Detailed diet compositions for the SAD, AID, and REG diets can be found in Tables 1, 2, and 3 respectively. Selected nutrient information is listed in Table 4.

Plantar Incision

Following 15 weeks of diet exposure, all remaining mice underwent a paw incision surgery as described by Brennan (26). Briefly, animals were anesthetized with isoflourane (2% in oxygen) and a 1 cm incision was made longitudinally through the skin and fascia of the hind paw. The underlying muscle was elevated and scraped using small curved forceps. Following incision, the wound was sutured and animals were placed in a clean cage atop a warming pad to recover from anesthesia. Following recovery, animals were returned to their home cage.

Flow Cytometry

Animals were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) and transcardially perfused with saline. Whole brains and spinal cords were harvested and enzymatically digested in 1mL of 1mg/mL of collagenase D (Sigma-Aldrich) and incubated in a 37°C water bath for 30 minutes, mixing every 5 minutes. Following enzymatic digestion, tissue was manually disrupted using a 1mL syringe plunger and single cell suspensions were created. Cells were F_C blocked then surface stained, and results were acquired using an Attune NxT Flow Cytometer (ThermoFisher Scientific) and analyzed with FlowJo software. The initial gating strategy involved an expanded FSC/SSC gate to isolate lymphocytes and a FSC-A/FSC-W gate to exclude doublets. Zombie Aqua fixable viability dye (BioLegend, San Diego, CA) was used to exclude dead cells. Fluorescence minus one controls and single stain controls were used to determine gate locations. A CD45-PE (clone: 30-F11)/CD11b-PE-Cy7 (clone: M1/70) gate was used to capture all myeloid cells (Supplemental Figures 1 and 2). Helper T cells were stained using CD3e-APC (clone: 145–2C11) and CD4-APC Fire750 (clone: RM4–5). Cytotoxic T cells were stained using CD3e-APC (clone: 145–2C11) and CD8-PerCPCy5.5 (clone: 53–6.7). B cells were stained with CD19-PacBlue (clone: 6D5; Supplemental Figure 1). Microglia were stained using a primary antibody stain against purinergic G-protein coupled receptor P2Y12 and a secondary anti-rabbit IgG-488 stain (Supplemental Figure 2). Fixation was performed with BD Cytofix (BD Biosciences). All antibodies were purchased from BioLegend with the exception of P2Y12, which was purchased from AnaSpec.

Statistics

Body weight was analyzed using a repeated measures ANOVA with Sex and Diet as between-subject variables and Time as the within-subject variable. Following a significant main effect of Sex, the data was split by Sex for further analysis. A multivariate ANOVA was used to analyze the immune cells with Sex, Diet and Day as between-subject variables. Data were split by tissue type (brain or spinal cord) and pre- or post-injury prior to the ANOVA. Following significant multivariate ANOVA, data were further split by Tissue Type, Injury, Day and Sex with Diet as the dependent variable. Bonferroni corrected post-hoc t-tests were used to compare the different diets post-injury. In all cases an alpha level of 0.05 was considered significant.

Results

Body Weight

There was a significant main effect of Time ($F(14, 1036) = 387.057, p < 0.001$) and a Time x Diet interaction ($F(14, 1036) = 7.863, p < 0.01$) with a Greenhouse-Geisser correction. There was a main effect of Sex ($F(1, 74) = 77.689, p < 0.001$) and Diet ($F(1, 74) = 15.682, p < 0.001$).

SAD-fed males gained more weight than REG-fed males ($F(1, 37) = 23.267, p < 0.001$). There was no effect of Diet for females (Figure 2).

Spinal Cords Pre-Injury

To assess acute and long-term exposure to the SAD on spinal cord infiltration, we compared REG-fed animals to SAD-fed animals at day 3 and week 15, prior to injury. Detailed information about cell counts is listed in Supplementary Table 1.

There was a main effect of Sex for CD3 ($F(1, 32) = 8.697, p < 0.01$), CD8 ($F(1, 32) = 6.294, p < 0.05$), CD11b ($F(1, 32) = 5.844, p < 0.05$), and CD45/CD11b ($F(1, 32) = 4.667, p < 0.05$). In general, males had a greater percentage of total cells. There was a main effect of Day for CD8 ($F(1, 32) = 11.177, p < 0.01$), CD19 ($F(1, 32) = 6.328, p < 0.05$), CD45/CD11b ($F(1, 32) = 12.533, p < 0.01$), and P2Y12 ($F(1, 32) = 11.252, p < 0.01$).

There were Sex by Day interactions in CD3 ($F(1, 32) = 12.426, p < 0.01$), CD8 ($F(1, 32) = 17.758, p < 0.001$), CD19 ($F(1, 32) = 11.301, p < 0.01$), CD11b ($F(1, 32) = 15.532, p < 0.001$), and P2Y12 ($F(1, 32) = 7.376, p < 0.05$). Prior to injury, there were no effects of diet on immune cells in the spinal cord.

Spinal Cords Post-Injury

To assess the effects of the SAD and the AID on spinal cord-immune cell entry after an injury, we compared immune cell infiltration in animals fed either the REG, SAD or AID at 3 and 7 days post paw incision. Detailed information about cell counts is listed in Supplementary Table 1.

There was a main effect of Sex for CD3 ($F(1, 46) = 32.309, p < 0.001$), CD4 ($F(1, 46) = 9.659, p < 0.01$), CD8 ($F(1, 46) = 11.074, p < 0.01$), and CD19 ($F(1, 46) = 51.837, p < 0.001$).

0.001). There was a main effect of Day for CD3 ($F(1, 46) = 43.151, p < 0.001$), CD4 ($F(1, 46) = 13.78, p < 0.01$), CD8 ($F(1, 46) = 15.705, p < 0.001$), CD19 ($F(1, 46) = 23.419, p < 0.001$), CD11b ($F(1, 46) = 12.456, p < 0.01$), CD45/CD11b ($F(1, 46) = 10.138, p < 0.01$), and P2Y12 ($F(1, 46) = 4.329, p < 0.05$). There was a main effect of Diet for CD4 ($F(2, 46) = 10.172, p < 0.001$), CD8 ($F(2, 46) = 3.905, p < 0.05$), and CD19 ($F(2, 46) = 9.101, p < 0.001$).

There was a significant Sex by Diet interaction for CD4 ($F(2, 46) = 7.434, p < 0.01$), CD8 ($F(2, 46) = 5.121, p < 0.05$), and CD19 ($F(2, 46) = 9.847, p < 0.001$). A significant Sex by Day interaction was found for CD3 ($F(1, 46) = 29.081, p < 0.001$), CD8 ($F(1, 46) = 7.949, p < 0.01$), CD19 ($F(1, 46) = 6.866, p < 0.05$), and P2Y12 ($F(1, 46) = 4.741, p < 0.05$). There was a significant Diet by Day interaction for CD4 ($F(2, 46) = 5.59, p < 0.01$). Lastly, there was a significant Sex by Diet by Day three-way interaction for CD4 ($F(2, 46) = 4.069, p < 0.05$).

There were no significant diet effects in females at any time-point. In males, significant diet effects emerged at both 3 and 7 days post-injury. Specifically, AID-fed males had fewer CD3⁺ T cells (Figure 3a), CD11b⁺ macrophages (Figure 3b), and CD45⁺/CD11b⁺ cells (Figure 3c) compared to SAD-fed males (p 's < 0.05) 3 days post-injury. SAD-fed males had significantly increased percentages of CD4⁺ T cells compared to REG- and AID-fed males ($p < 0.05$; Figure 4a) 7 days post-injury. In addition, AID-fed males had fewer CD19⁺ B cells than both REG- and SAD-fed males ($p < 0.05$; Figure 4b) 7 days post-injury. Together, these data suggest that the SAD led to increased infiltration of immune cells (CD4⁺ T cells) after injury, and the increase could be prevented with a switch to the AID.

Brains

To examine if long-term SAD-consumption led to immune cell entry into the brain, SAD-fed and REG-fed animals were compared after 15 weeks of diet consumption.

There was a main effect of Sex for CD3 ($F(1, 8) = 14.847, p < 0.01$), CD8 ($F(1, 8) = 6.015, p < 0.05$), CD19 ($F(1, 8) = 24.814, p < 0.01$), and CD45/CD11b ($F(1, 8) = 10.814, p < 0.05$). In general, males had a greater percentage of total cells. There were main effects of Day for CD3 ($F(1, 8) = 24.212, p < 0.01$), CD4 ($F(1, 8) = 6.252, p < 0.05$), CD8 ($F(1, 8) = 14.905, p < 0.01$), and CD19 ($F(1, 8) = 13.651, p < 0.01$).

There were significant Sex by Day interactions for CD3 ($F(1, 8) = 18.146, p < 0.01$), CD4 ($F(1, 8) = 21.172, p < 0.01$), CD8 ($F(1, 8) = 8.406, p < 0.05$), CD11b ($F(1, 8) = 12.54, p < 0.01$), and CD45/CD11b ($F(1, 8) = 8.351, p < 0.05$).

Specifically, SAD-fed males had increased levels of CD45⁺/CD11b⁺ cells (Figure 5a) and CD11b⁺ macrophages (Figure 5b) compared to REG-fed males. There were no effects of diet on the number of microglia in the brain prior to or following injury in either sex. There were no significant effects of diet on immune cells in female brains.

Discussion

In the present study we determined that long-term SAD consumption led to an increase in macrophages in the brains of male mice in the absence of injury. No diet effects were seen in microglia in male mice. Following injury, AID-fed males had fewer T cells (CD3⁺) and macrophages within the spinal cord at day 3, as compared to SAD-fed males. Additionally, SAD-fed males had an increase in T helper cells within the spinal cord 7 days after injury that was completely prevented by the AID. AID-fed males also had significantly fewer B cells in the spinal cord at 7 days post-injury compared to both REG- and SAD-fed males. These results demonstrate the presence of SAD-induced changes in leukocyte composition in male mice within the CNS and further illustrate that the AID can be used to counteract those effects. In contrast, female mice had no diet-induced changes in any cell type examined, in either the brain or spinal cord. The difference in response to SAD administration in males versus females may be due to the fact that only male mice showed excess weight gain on the SAD versus REG diet. Alternately, differential response to diet could be due to other factors such as hormonal variations that were not examined here.

Obesity is a global health concern that is often associated with cardiovascular and metabolic complications. It is now accepted that obesity is also characterized by persistent low-grade inflammation. One serious side effect of this inflammation is chronic pain. Obese individuals are more susceptible to chronic pain (3) and the two conditions often occur in parallel.

A major contributor in the development of obesity is a poor-quality diet. It has been demonstrated that various components of a poor diet, such as carbohydrates (27–29), saturated fats (30, 31), and high levels of omega-6 fatty acids (32, 33) are pro-inflammatory. There is also evidence in rats that a high-fat diet leads to pain in the absence of obesity (34). In line with this, our previous study in rats showed that a diet effect was present even when the rats were not obese (7). Therefore, independent of obesity, a poor diet can lead to immune activation and subsequent pain. In the current study, both the SAD and AID are high-fat diets. However, the fat sources in the two diets are distinctly different with the SAD being primarily comprised of trans and saturated fats with a high omega-6 to omega-3 ratio. This is compared to the AID which contains flaxseed oil to increase the omega-3 content. Our findings in which these two high-fat diets lead to different results suggests that the source of fat, and not the absolute amount of fat, may be the primary determinant of dietary effects on pain and inflammation. It should be noted that trans fats have very recently been banned by the US Food and Drug Administration, but are expected to remain in circulation until 2020. Therefore, our SAD is a model of the *current* American diet, but future iterations will require elimination of trans fat.

There is evidence that sex differences exist in the mechanism of pain (35). Specifically, it has been shown that mechanical sensitivity following nerve injury is mediated by microglia in male mice, whereas females rely on T cells for the maintenance of chronic pain. Interestingly, despite this difference, both sexes have similar microglial activation following nerve injury (22) and chronic constriction injury (36). This is in line with our previous results in which the SAD led to an increase in microglia activation within the spinal cord of

both male and female rats (7). In contrast to these findings, the present study found an effect of diet exclusively in males with no effect of diet on microglia. One reason for this difference may be in the methods used to determine the presence of immune cells. Microglia are typically measured via immunohistochemistry. However, the common microglial marker Iba-1 also stains macrophages. Therefore, in order to identify the distinct cell types, flow cytometry was used with the microglia specific marker P2Y12. Additionally, with immunohistochemistry only certain sections are being analyzed giving more of a snapshot of what is occurring. In the present study, whole spinal cord samples were used to gain an overall picture of immune cell contributions.

Multiple studies have uncovered the contribution of T cells in the mechanism of neuropathic pain. One group found an increase in T cell infiltration into the spinal cord following a nerve injury (24). Of note, T cell deficient animals did not develop mechanical allodynia following injury, indicating a clear role for T cells in injury-induced hypersensitivity. Several studies found that T helper cells infiltrate the spinal cord in a neuropathic pain model and are involved in the subsequent hypersensitivity (37–39). This finding has been validated using CD4 deficient animals in which mice lacking CD4⁺ T cells did not experience spinal cord infiltration following a neuropathic pain model compared to wild type animals (37). Recently, the role of T cell infiltration in a model of inflammatory pain was investigated. Flow cytometry revealed an increase in spinal cord-infiltrating T cells following an inflammatory injury (40). Together, these studies indicate a clear role for T cell infiltration in the development of various types of pain. Our results support previous reports of increased T cell infiltration into the spinal cord following injury and demonstrate that a change in diet can counteract that increased infiltration.

Previous studies in our lab have demonstrated that our novel SAD can lead to prolonged recovery and immune activation both peripherally and centrally in both male and female animals (7). Additionally, our lab has shown that an anti-inflammatory diet not only prevents the negative consequences of the SAD, but if used as an intervention can reverse the effects (8). However, the effect of SAD on T cells had not been determined.

The present study aimed to examine T cell infiltration into the spinal cord of mice following a plantar incision model of post-surgical pain following prolonged diet consumption. We were interested in what effects acute and long-term diet exposure would have on immune cell infiltration in mice. We found that there were no significant effects of a poor diet on T lymphocytes within the spinal cord at 3 days or 15 weeks of diet consumption. This suggests that in the absence of an insult, acute or long-term SAD consumption does not lead to spinal cord T cell infiltration. Alternatively, 7 days following paw incision there was a significant increase in CD4⁺ T cells in male mice who consumed the SAD. This supports the work of others in which an increase in CD4⁺ cells were present at 7 days post-injury (40). Additionally, this suggests that the SAD leads to greater T cell infiltration in the presence of an injury compared to the AID and REG diets.

To determine if this effect could be prevented, at the time of injury, a subset of SAD-fed mice had their diets switched to the AID. Remarkably, the increase in CD4⁺ cells 7 days after injury was prevented by the AID. At 3 days following injury, AID-fed males had

significantly less CD3⁺ (total T cells) cells compared to SAD-fed males. In addition to T cells, we also found that the AID led to significantly less CD45⁺CD11b⁺ cells and CD11b⁺ macrophages at 3 days following injury, and less CD19⁺ B cells at 7 days following injury in male mice. This immune cell effect of the AID seems to coincide with the enhanced recovery when the AID is used as an intervention in our previous work (8).

As previously stated, a novel flow cytometry protocol was utilized to differentiate microglia from macrophages, among other cell types. Microglia and macrophages both express CD11b and CD45 on their cell surface. Traditionally, in order to distinguish between the two cell types, the expression levels of the two markers are considered. For example, both cell types have similar CD11b expression, but macrophages are deemed to have a high CD45 expression level, whereas microglia have an intermediate level of CD45 expression (41–43). Therefore, cells that are CD45^{hi}CD11b^{hi} are typically considered as macrophages, while cells that are CD45^{lo}CD11b^{hi} are microglia. However, in the current study, we utilized the microglial specific marker, P2Y12, in order to differentiate these cells from macrophages (44). In microglia, P2Y12 activation in response to sensing damage within the CNS leads to chemotaxis. P2Y12 receptors are highly expressed in ramified microglia and are down-regulated following activation (45). In the current study, there were no differences seen in P2Y12⁺ microglia in the spinal cords or brains at any time-point. Due to the downregulation in P2Y12 after microglial activation, we would not expect to see significant increases in P2Y12-positive cells in the SAD animals. However, it stands to reason that a portion of the CD45⁺/CD11b⁺ cells may be activated microglia that are no longer expressing P2Y12. Further research is needed to distinguish activated microglia from infiltrating macrophages within the CNS. Our results suggest that our SAD does have an effect on CD45⁺/CD11b⁺ macrophage/microglia infiltration into the spinal cord in male mice and that the AID can prevent the SAD-induced increase in infiltration.

These findings support our behavioral data in which SAD-fed mice have prolonged recovery that is prevented by switching mice to the AID. The current data suggests that the behavioral effects seen may be due to immune modulation by the different diets. These results support the use of an anti-inflammatory diet as an alternative, non-pharmacological treatment for pain and inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Long-term SAD consumption increased macrophages in the brain.
- SAD consumption increased T cell infiltration into the spinal cord following injury.
- AID intervention at injury prevented the SAD-induced changes in spinal cord.

Figure 1:

Experimental Design. Following 2 weeks of regular diet, mice were divided into either the SAD group, or maintained on the REG diet. A subset of animals were sacrificed at day 3 and week 15. After 15 weeks, all remaining mice underwent a paw incision surgery and a subset of SAD animals were switched to the AID for the remainder of the experiment. Subsets of mice were sacrificed 3 and 7 days following injury.

Figure 2: Weight changes during diet exposure. Body weight (in grams) for male (squares) and female (circles) mice over 15 weeks. *** $P < .001$. All data expressed as mean $\pm$ SEM.

Figure 3:

Effects of diet on immune cells within the spinal cord of males 3 days post-injury.

Percentage of (A) CD3⁺ T cells, (B) CD11b⁺ macrophages, and (C) CD45⁺/CD11b⁺ cells as determined by flow cytometry. *P < .05. All data are expressed as mean ± SEM.

Figure 4:
Effects of diet on immune cells within the spinal cord of males 7 days post-injury.
Percentage of (A) CD4⁺ T cells and (B) CD19⁺ B cells as determined by flow cytometry. *P < .05 **P < .01. All data are expressed as mean ± SEM.

Figure 5: Effects of diet on immune cells within the brain of male mice. Percentage of (A) CD45⁺/CD11b⁺ cells and (B) CD11b⁺ macrophages as determined by flow cytometry. *P < .05. All data are expressed as mean ± SEM.

Table 1:

Composition of the Standard American Diet. Amounts are given as dry weight where appropriate.

Ingredient	g/kg
Casein	141.0
L-Cystine	1.8
Bleached White Flour	410.0
Corn Starch	25.0
Maltodextrin	60.0
Sucrose	126.8
Soybean Oil	17.3
Corn Oil	10.6
Cottonseed Oil	20.9
Lard	18.0
Beef Tallow	16.0
Anhydrous Milkfat	23.3
Vegetable Shortening, hydrogenated (Primex)	59.5
Cholesterol	0.4
Cellulose	19.0
Mineral Mix, nTWD (110422)	35.0
Sodium Chloride	4.0
Vitamin Mix, nTWD (110423)	10.0
Choline Bitartrate	1.4
Antioxidant, TBHQ	0.03

Table 2:

Composition of the Anti-inflammatory Diet. Amounts are given as dry weight where appropriate.

Ingredient	g/kg
Whey Protein Isolate	218
L-Cystine	1.8
High Amylose Corn Starch	337.0188
Maltodextrin	170
Flaxseed Oil	8.5
Soybean Oil	171.5
Cellulose	50
Mineral Mix, w/o Ca & P	14.4
Calcium Phosphate, dibasic	13.16
Calcium Carbonate	2.5
Vitamin Mix	10
Choline Bitartrate	2.5
Antioxidant	0.34
EGCG	0.12
Sulforaphane	0.0006
Resveratrol	0.0006
Curcumin	0.1
Ginseng	0.06

Table 3:

Composition of the Regular Chow Diet. Amounts are given as dry weight where appropriate.

Ingredient	g/kg
Fish meal (60% protein)	90
Soybean meal (48.5% protein)	50
Alfalfa meal (17% protein)	20
Corn gluten meal (60% protein)	20
Ground whole hard wheat	355
Ground #2 yellow shelled corn (8.5% protein)	210
Ground whole oats	100
Wheat middlings	100
Brewers dried yeast	10
Soy oil	15
Salt	5
Dicalcium phosphate	15
Ground limestone	5
Premixes	5

Table 4:

Macronutrient content for each experimental diet. Data represented as the percentage of kilocalories from each macronutrient.

Macronutrient	% kcal from		
	SAD	AID	REG
Protein	15.4	20.1	24
Carbohydrate	49	40	62
Fat	35.6	39.9	14