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Influence of Dietary Modification and/or Exercise on Markers of Inflammation in Male Rats

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INFLUENCE OF DIETARY MODIFICATION AND/OR EXERCISE ON MARKERS OF INFLAMMATION IN MALE RATS

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ABSTRACT

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The goal of the investigation was to examine the influence of a dietary intervention, with/without exercise, on markers of inflammation in plasma. Male Long-Evans rats (n = 28, aged 3-4 weeks) were assigned to either a "Daniel Fast" or "Western Diet" with/without exercise training for 13 weeks. Regular exercise training was performed three days a week. Following the 13-week intervention, the animals were sacrificed, followed by blood collection. Cytokines were measured via a magnetic bead panel assay. No statistical significance was found between groups ($p > 0.05$); however, a pattern was noted for both IL-1β and IL-10. Body weight gain was greater in WD vs. all other groups. Body composition demonstrated a lower fat mass in DF groups vs. WD groups and no difference between groups in lean mass. These data indicate that the DF, coupled with exercise, may produce favorable results concerning health and inflammatory status compared to the WD.

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Background

Inflammation is typically described as either acute or chronic. Acute inflammation refers to the body's response to pain or injury and is an important component involved in the healing process (Hotamisligil, 2006; Punchard, Whelan, & Adcock, 2004). The present research focused on chronic inflammation which is indicated by repeatedly elevated circulating inflammatory markers and its association with obesity and multiple diseases. Recent studies have investigated elevated concentrations of specific inflammatory proteins such as tumor necrosis factor-alpha (TNF-α) (Lakhdar et al., 2013; Saghizadeh, Ong, Garvey, Henry, & Kern, 1996; Sethi & Hotamisligil, 1999; Spaulding, Walford, & Effros, 1997), interleukin-6 (IL-6) (Lakhdar et al., 2013; Strohacker, Wing, & McCaffery, 2013; Thompson et al., 2010), interleukin-1beta (IL-1β) (Caughey, Mantzioris, Gibson, Cleland, & James, 1996; Nov et al., 2013; Wieser, Moschen, & Tilg, 2013) and C-reactive protein (CRP) (Lagrand et al., 1999; Lucas-Luciardi et al., 2012; Pradhan, Manson, Rifai, Buring, & Ridker, 2001; Puglisi & Fernandez, 2008) in an attempt to determine the relationship between disease (e.g., obesity) and markers of tissue inflammation. Several studies have examined adipose tissue and determined that it is the primary site of secretion for a variety of inflammatory proteins that have systemic effects (Cildir, Akıncılar, & Tergaonkar, 2013; Giannopoulou et al., 2005; Poitou, Dalmas, & Clément, 2013; Thompson et al., 2010).

Physical inactivity and the consumption of a diet high in saturated fat and sugars increase chronic inflammatory levels (Lakhdar et al., 2013; Rocha & Libby, 2009; Thompson et al., 2010). Specifically, recent research has studied the anti-inflammatory effect of exercise (Kasapis & Thompson, 2005; Lakhdar et al., 2013; Petersen &

Pedersen, 2005; D. Thompson et al., 2010), noting significant decreases in multiple inflammatory markers. Partaking in a diet devoid of saturated fat and simple sugar has resulted in similar findings (Alleman, Harvey, Farney, & Bloomer, 2013; Bloomer et al., 2010; Lakhdar et al., 2013). Exercise and diet in unison have been investigated to a lesser extent, as pertaining to the impact on long-term inflammatory levels (Lakhdar et al., 2013; Vieira, Valentine, Wilund, & Woods, 2009).

The health implications of "Western Dieting" are of major concern within the research and medical fields (Grotto & Zied, 2010). This is because the typical Western Diet consists of high calorie loads, is rich in saturated fats, is dense in refined carbohydrates and is high in sodium and cholesterol (Biondi-Zoccai, Abbate, Liuzzo, & Biasucci, 2003; Bulló, García-Lorda, Megias, & Salas-Salvadó, 2003; Cordain et al., 2005). Such a dietary intake leads to increased inflammation and is correlated with hypertension (Galland, 2010), hyperglycemia (Gregersen, Samocha-Bonet, Heilbronn, & Campbell, 2012), dyslipidemia (Esteve, Ricart, & Fernandez-Real, 2005) and a myriad of other health-specific problems (Burgmaier et al., 2010; Cordain et al., 2005). The primary outward sign of routine following of the Western Diet is an increase in adipose tissue (Galland, 2010; G. S. Hotamisligil, 2006). This occurs around the abdominal region as visceral adipose tissue, one of the primary sites involved in inflammation (Berg & Scherer, 2005). Although acute inflammation can be beneficial in an attempt to promote healing (G. S. Hotamisligil, 2006), chronic inflammation can be pathogenic (Cordain et al., 2005). Many diseases such as diabetes (Biondi-Zoccai, Abbate, Liuzzo, & Biasucci, 2003; F. Cosentino & Egidy Assenza, 2004; Dandona, Aljada, & Bandyopadhyay, 2004; Evans, Goldfine, Maddux, & Grodsky, 2002; Wellen &

Hotamisligil, 2005), cardiovascular disease (Berg & Scherer, 2005; R. Bloomer et al., 2010; Burgmaier et al., 2010; Hilfiker-Kleiner, Landmesser, & Drexler, 2006), cancer (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009; Mantovani, Allavena, Sica, & Balkwill, 2008), and sarcopenia (Hsiao et al., 2009; Rieu et al., 2009) have been linked to chronic low-grade inflammation in humans and animals. Moreover, all of the aforementioned diseases have increased dramatically in the United States over the last few decades (Allison, Fontaine, Manson, Stevens, & VanItallie, 1999), in a similar rise as has been observed for obesity.

Attempts have been made to lessen inflammation, with one such attempt focused on reducing caloric intake (Alleman et al., 2013; Bloomer et al., 2010; Chiba & Ezaki, 2010; Craig, 2010; Galland, 2010; Varady & Hellerstein, 2007) — in which investigators have utilized numerous dietary protocols. For example, Lakhdar et al. set the protocol at 500 kcal/day below initial dietary assessments in human subjects (Lakhdar et al., 2013) and protocols have involved as much as a 50% reduction in kcal intake using animal models (Chiba & Ezaki, 2010). In general, studies have noted significant reductions in plasma TNF-α and IL-6 concentrations.

Aside from the above mentioned dietary approaches that restrict calories, a restriction model that does not limit calories but does limit the types of foods consumed has been recently investigated (Alleman et al., 2013; Bloomer et al., 2010). This plan, known as "The Daniel Fast", due to its origin from the Biblical Book of Daniel, is a stringent vegan diet that prohibits all animal products, sweeteners (natural and unnatural), processed foods, flavorings, preservatives, additives, alcohol and caffeine. Fruits, vegetables, legumes, whole grains, nuts, seeds and oil may be eaten ad libitum while

following the Daniel Fast (Alleman et al., 2013; Bloomer et al., 2010). The effectiveness of the Daniel Fast to lower systemic inflammation has been reported in prior studies, when compared to subjects' pre-fast inflammatory levels when following a typical Western Diet. Specifically, reductions in both CRP and white blood cell levels have been noted (Alleman et al., 2013; Bloomer et al., 2010). These findings are similar to what have been reported in other studies in which vegan diets have been investigated (Craig, 2010; McCarty, Barroso-Aranda, & Contreras, 2009).

Various tissues (e.g., blood, adipose, skeletal muscle, cardiac muscle, liver) can present with elevated degrees of inflammation—levels that may precipitate disease (Giannopoulou et al., 2005; Lorentz, Schwengberg, Sellge, Manns, & Bischoff, 2000; Marcell, McAuley, Traustadóttir, & Reaven, 2005; Thompson et al., 2010). To our knowledge, these tissues have not yet been examined following a vegan diet and certainly not following a Daniel Fast dietary model in regards to inflammatory status. The aforementioned dietary plans have been investigated using human subjects, with systemic inflammatory biomarkers being the lone outcome measure (Alleman et al., 2013; Bloomer et al., 2010; Craig, 2010; McCarty et al., 2009). Inclusion of tissue aside from blood should provide more information on the role of both exercise and dietary intake to modulate inflammation over time if results from blood samples warrant further investigation. Utilizing a rodent model allows for complete dietary control and more invasive procedures such as tissue and organ extraction otherwise unachievable in the human model because of ethical considerations.

Specific to the above, past studies have evaluated circulating inflammatory cytokines and their link to disease (Lakhdar et al., 2013; Strohacker et al., 2013;

Thompson et al., 2010). Tumor-necrosis-factor-α, Interleukin-6, Interleukin-10, and Interleukin-1β are among the most highly examined inflammatory cytokines (Burgmaier et al., 2010; Fichorova et al., 2008; Hotamisligil, Arner, Caro, Atkinson, & Spiegelman, 1995). Examination of these cytokines in varying tissue samples, via biochemical assay-based measures, would undoubtedly provide significant advances in this area of research, providing results from blood samples warrant further investigation.

Based on the above, the purpose of the proposed investigation is to examine the effect of a Western Diet or Daniel Fast intervention (over a 13-week period), with and without aerobic exercise, on selected markers of inflammation in blood plasma samples. It is possible that the combination of regular exercise training and dietary intake in accordance with the Daniel Fast plan regimen may significantly reduce inflammation, which may have implications for lowering disease risk over the lifespan. If differences in inflammatory markers are noted in plasma samples, other tissues may be investigated to more fully elucidate the influence of diet and exercise on chronic inflammation.

Methods

Overview of Experimental Design

Male Long-Evans rats (n = 28, aged 3-4 weeks) were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Upon arrival to the facility, all rats were individually housed at the animal care center on The University of Memphis main campus. Animals were initially maintained on a standard 12:12-h light-dark cycle. Animals had a two week acclimation to the animal care facility. Acclimation included familiarization to the cage, to the assigned diet (using a combination of the assigned diet and standard rat chow, in various proportions), and to the handlers. In addition, animals

underwent familiarization to the treadmill consisting of three separate days (i.e., walk on treadmill for 5 minutes at 20 m/min). The animals were smoothly transitioned to a 12:12-h light-dark cycle (3:00am/3:00pm) during this two-week time period.

Although our prior work with the Daniel Fast has exclusively involved human subjects, animals were used in the present design for the following reasons. First, using rats provided us with the ability to maintain control of all key variables known to impact the inflammatory response (i.e., sleep, type and volume of food, volume of exercise, intensity of exercise, health status, genetics). While recommendations can be made to humans to abide by our guidelines, these variables cannot be controlled with precision, as can be done in rats. Second, it was our belief that using rats would reduce variability in response, and was expected that a homogenous sample of animals would exhibit less variability in inflammatory measures as compared to human subjects. Follow-up work using human subjects and in multiple tissues may be warranted.

Dietary and Exercise Intervention

Following baseline assessments the animals were randomly assigned to one of four intervention groups: Western Diet with exercise (n = 7); Western Diet without exercise (n = 7); Daniel Fast with exercise (n = 7); Daniel Fast without exercise (n = 7). Both diets (provided in pellet form) were purchased from Research Diets, Inc. (New Brunswick, NJ). The Western Diet is a standard product produced to mimic a typical human Western diet, containing 17% protein, 43% Carbohydrates, and 40% Fat. Custom rat chow was used to replicate the "cleanliness" of the Daniel Fast plan, including a macronutrient breakdown of approximately 15% protein, 60% Carbohydrates, and 25% Fat. The specific nutrient breakdown of each plan is provided in Table 1.

The dietary intervention period was approximately 13 weeks in duration. In all groups, the animals were allowed to feed *ad libitum*. Water was also allowed *ad libitum*. Weekly body weights were measured and recorded.

In addition to the two different dietary regimens, animals were assigned to either exercise or no exercise. Animals in the no exercise group were placed on the treadmill daily for a period of 5 minutes, simply to allow for familiarization to the apparatus. Animals in the exercise group performed endurance exercise on a motorized treadmill three days per week for the 13 week intervention. The speed and duration was progressively increased. Specifically, the animals began training at 20 m/min for 15 min/day (week 1), progressed to 25 m/min for 30 min/day (week 2), and 25 m/min for 35 min/day (weeks 3-13). Progression of this sort is typical in animal training studies (Jin et al., 2000).

Blood Sample Collection

After euthanasia via CO_2 inhalation, the thoracic cavity of each rat was exposed and blood samples were immediately collected from the inferior vena cava in vacutainers containing EDTA and centrifuged for 15 minutes at 2000xg. Plasma samples were then collected and stored at -70°C. The collection, centrifugation, and storage methods were conducted in a manner similar to that of Vieira et al. (2009).

Biochemical Analysis

A Milliplex® Map Kit was purchased from the EMD Millipore Corporation (Darmstadt, Germany) to analyze cytokines of interest in rat samples. Specifically, antibody-immobilized magnetic beads were purchased for the following analytes of interest: IL-1α, IL-4, IL-1β, IL-6, IL-13, IL-10, IFNγ, and TNF-α. The rat

cytokine/chemokine magnetic bead panel allowed for a more time and cost efficient one-plate analysis rather than purchasing several ELISA kits specific to each cytokine of interest. The guidelines provided within the kit for plasma samples were followed precisely and results were obtained the day the immunoassay procedure was performed. The plate was read immediately upon finishing the assay on MAGPIX®, using xPONENT software, at the VA Medical Center (Memphis, TN). All samples were analyzed in duplicate.

Body Composition

All animals underwent a Dual Energy X-ray Absorptiometry (DXA) exam following the intervention—approximately one week before blood sample collection. This was done to examine potential post-intervention differences in body composition among groups. No DXA exam was performed pre-intervention. Animals were anesthetized for approximately 10 minutes (using isoflurane) to allow the scan to be performed.

Statistical Analysis

Biochemical analysis of blood samples via a Milliplex® Map Kit provided data specific to each inflammatory variable of interest. DXA scans provided data pertaining to body composition. All data are expressed as the mean ± standard error. A one-way analysis of variance was used to analyze each variable tested, with an alpha level of significance set at 0.05.

Results

A total of 27 animals completed all aspects of this study. One animal in the WD+E group died during week 2 of the intervention – approximately 30 minutes following the exercise training session. Upon examination by the University veterinarian, it was noted that the animal's abdomen was filled with blood, with a suspected aneurism or tear in liver. All other animals successfully completed the intervention.

Results from the assay for the specific cytokines of interest are presented in Table 2. The concentrations of four of the cytokines measured were below the limit of detection and are not included in the table. Although there were differences suggesting a distinct pattern between groups for IL-1β and IL-10 (Figure 1), no significant differences were found ($p > 0.05$). Of the remaining six cytokines of interest, no significant differences were noted ($p > 0.05$).

Several differences were noted for anthropometric measurements. For example, body weight displayed a group effect (WD > all other groups) ($p < 0.0001$) and a time effect: pre to post (WD+E 177 % ↑, WD 205 % ↑, DF+E 148 % ↑, DF 168 % ↑) ($p < 0.0001$); as well as a group x time interaction effect ($p < 0.0001$). Body weight gain was greater in WD vs. all other groups ($p < 0.05$). There was no significant difference between DF groups and WD+E for body weight gain ($p > 0.05$).

A group effect was noted for mean fat mass ($p < 0.0001$), with DF groups lower than WD groups; WD+E was lower than WD ($p < 0.05$). A group effect was noted for body fat percentage ($p < 0.0001$), with DF groups lower than WD groups; WD+E was

lower than WD ($p < 0.05$). There was no significant difference in lean mass between groups ($p = 0.14$). Anthropometric data for all animals are presented in Table 3.

Discussion

This was the first study to our knowledge that investigated the combined influences of exercise and the Daniel Fast in relation to inflammatory status. The main findings from this study are as follows: 1) no statistically significant differences were detected among groups for the eight measured cytokines, 2) differences indicating a distinct pattern were noted among all four groups in relation to IL-10 and IL-1β concentrations (Figure 1), 3) body weight gain was greater in WD vs. all other groups; no difference was noted between DF groups and WD+E (Table 3), and 4) body composition analysis demonstrated a lower fat mass and percent body fat in DF groups vs. WD groups, with no difference in lean mass (Table 3).

Although no differences of statistical significance were found among groups, a distinct pattern was noted between the four groups for both IL-10 and IL-1β concentrations. Lack of statistical significance may be attributed to several factors that could have impacted the results in this study. For example, due to the relatively small sample size for each group, statistical power was minimized and outliers had a higher impact on group mean and variability, thus increasing standard error. In future studies, a larger sample size may increase statistical power and provide investigators with the ability to note differences between groups. This is suggested based on the predictive pattern seen with such a small number of samples in the current study.

In addition, the young age of the rats in this study may have affected the expression of several important inflammatory cytokines that may not be fully manifested

until later in life (Kiecolt-Glaser et al., 2003; Michaud et al., 2013; Moon et al., 2012). This may help to explain why several of our measurements were too low to detect. Future longitudinal studies or studies incorporating rats of varying age groups may prove to be beneficial in understanding the roles of major inflammatory cytokines over the life span rather than specifically at a younger age.

The number of IL-10 and IL-1β sample results obtained for each group were as follows: IL-10-WD+E-6, WD-7, DF+E-7, DF-7 and IL-1β-WD+E-6, WD-7, DF+E-6, DF-5. The WD+E group had one less sample due to the rat that died during training and also had an outlier that highly impacted the overall mean for both IL-10 and IL-1β concentrations. Discounting this outlier, a pattern of WD > WD+E > DF > DF+E was noted between groups.

These results indicate that following a Daniel Fast intervention and incorporating endurance exercise may help to maintain lower levels of IL-1β over time as compared to all other groups. This is important as IL-1β is secreted by all nucleated cells including macrophages, monocytes, B cells, fibroblasts, chondrocytes and keratinocytes (Huang, Huang, & Chen, 2009) and has been repeatedly labeled as one of the most significant factors in regulating both local and systemic onset of acute and chronic inflammation (Besedovsky, del Rey, Sorkin, & Dinarello, 1986; Coppack, 2001; Huang et al., 2009; Permana, Menge, & Reaven, 2006). The pattern found between groups also confirms previous research indicating that physical inactivity coupled with consuming a WD leads to higher inflammatory levels (Lakhdar et al., 2013; Rocha & Libby, 2009; D. Thompson et al., 2010).

The DF+E group also displayed the lowest levels of IL-10. Research has demonstrated the inhibitory property of IL-10 in regard to the release of many pro-inflammatory mediators such as TNF-α, IL-6 and IL-1β and enhanced release of anti-inflammatory mediators (Chang et al., 2013; Huang et al., 2009; Sabat et al., 2010). In individuals with irregular concentrations of IL-10, the inflammatory response is either constantly activated or suppressed. This may best be demonstrated in individuals with a relative or absolute IL-10 deficiency resulting in IBS and in certain autoimmune diseases, as well as in IL-10-over-expressing animals (Groux et al., 1999; Sabat et al., 2010). It can therefore be understood as to why the DF+E group also displayed the lowest levels of IL-10, while the WD group displayed the highest levels of IL-10.

As seen in Figure 1, IL-10 concentrations between groups were similar to IL-1β concentrations. Based on previous research, this may best be explained as a compensatory effect, although the exact molecular mechanisms of immunosuppressive effects of IL-10 on APCs and T cells are still not completely understood (Sabat et al., 2010). These findings are consistent with studies examining human populations investigating various diseases in humans such as malaria and have found that IL-10 levels are dependent upon disease severity (pro-inflammatory status) (Lyke et al., 2004). Similar results have been reported concerning IL-10 and IL-1β levels in adolescents. One recent study reported a decline in IL-10 concentration in overweight and obese adolescents and suggested this may further contribute to the IL-1β-mediated inflammatory environment associated with obesity (Chang et al., 2013). This would indicate that disease severity, rather than age, would play a more significant role in

influencing IL-1β and IL-10 concentrations. Future studies need to be conducted in this area of research to gain further insight into pro-/anti-inflammatory cytokine relationships.

Four of the eight cytokines measured had undetectably low levels. A recent study investigated TNF-α concentrations in the serum of both young and old Fischer rats in response to chronic endurance exercise over a period of 12 weeks (Moon et al., 2012). Results from this study indicate that endurance exercise lowered the TNF-α concentration in young rats (3-6 months) and did not affect concentrations in older rats (22 months). This may partly explain why TNF-α levels in the present study were so low and often undetectable. Because rats in the present study were only 3-4 months of age at the time of blood collection, and were also exposed to a similar training protocol of the same length, low overall values for this measure were noted. The previously mentioned study also measured IL-6 concentrations between the two age groups in response to chronic endurance exercise and noted significant changes within hepatic cells. Therefore, a possible explanation is that because IL-1β acts as one of the main initial activators of the inflammatory response, and IL-10 acts as a primary anti-inflammatory mediator—while many of the later stage cytokines are undetectable, as they are not yet produced in the inflammatory response at the age being investigated.

The macronutrient content between the two diets is vastly different and significant differences between groups were noted concerning body weight and composition that parallel IL-1β and IL-10 concentrations. A diet high in simple carbohydrates and saturated fat coupled with a sedentary lifestyle (WD) displayed the highest weight gain and percentage of body fat as compared to all other groups. This can be attributed to both inactivity (Ekelund, Brage, Besson, Sharp, & Wareham, 2008) and dietary composition

(Milagro, Campión, & Martínez, 2006). Previous research in humans has found that partaking in a Daniel Fast for a period of three weeks has highly beneficial outcomes concerning multiple health parameters, including lowered CRP levels, WBC, cholesterol, insulin, and blood pressure (Alleman et al., 2013; Bloomer et al., 2011; Bloomer et al., 2010). This is primarily due to a lowered consumption of saturated fats and refined carbohydrates and increased consumption of complex carbohydrates (and fiber) and polyunsaturated fats.

Interestingly, both WD groups displayed higher percentages of body fat than the DF groups. This is in accordance with higher IL-1β and IL-10 levels found during this study. Past research indicates favorable outcomes in human subjects while adhering to the DF, as pertaining to overall body mass and body fat content (Bloomer et al., 2011; Bloomer et al., 2010). These findings verify that consuming a WD while maintaining low levels of physical activity can lead to a higher body fat content. Further, results indicate that regardless of physical activity level, consuming a WD does not provide the benefits that a traditional DF may provide concerning overall body content.

Conclusion

To our knowledge, this is the first study to assess chronic inflammatory status in relation to the Daniel Fast diet with and without exercise training. Our results indicate a distinct pattern between groups indicative of the potential benefits that the DF diet may provide concerning inflammatory levels as compared to a WD, when coupled with regular exercise training. The lower levels of IL-1β and IL-10 may be attributed to the macronutrient mix (low glycemic carbohydrate, high polyunsaturated fat), coupled with the drastically lower body fat levels (exercise- and diet-induced), as compared to the WD

animals. Based on our results, further investigation of the effects of a DF diet coupled with physical performance on markers of inflammation in human subjects is warranted.

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APPENDIX A – TABLES/FIGURES

Table 1. Dietary Nutrient Breakdown

Nutrient	**Western Diet**		**Daniel Fast**	
	gm %	kcal %	gm %	kcal %
Protein	20	17	15	15
Carbohydrate	50	43	58	59
Fat	21	40	11	25
Fiber	5	0	13	1
Total		100		100
kcal/gm	4.7		3.9	
Casein	195	780	0	0
Soy Protein	0	0	170	680
DL-Methionine	3	12	3	12
Corn Starch	50	200	0	0
Corn Starch-Hi Maize 260 (70 % Amylose and 30 % Amylopectin)	0	0	533.5	2134
Maltodextrin 10	100	400	150	600
Sucrose	341	1364	0	0
Cellulose, BW200	50	0	100	0
Inulin	0	0	50	50
Milk Fat, Anhydrous	200	1800	0	0

Corn Oil	10	90	0	0
Flaxseed Oil	0	0	130	1170
Ethoxyquin	0.04	0	0.04	0
Mineral Mix S1001	35	0	35	0
Calcium Carbonate	4	0	4	0
Vitamin Mix V1001	10	40	10	40
Choline Carbonate	2	0	2	0
Ascorbic Acid Phosphate, 33% active	0	0	.41	0
Cholesterol	1.5	0	0	0
Total	**1001.54**	**4686**	**1187.95**	**4686**
Saturated g/kg	122.6		7.8	
Monunsaturated g/kg	60.2		19.7	
Polyunsaturated g/kg	13.5		77.7	
Cholesterol mg/kg	2048		0	
Saturated % Fat	62.4		7.4	
Monunsaturated %Fat	30.7		18.7	
Polyunsaturated %Fat	6.9		73.9	
Ascorbic Acid mg/kg	0		114	

Table 2. Cytokine concentrations of male rats assigned to two different diets with and without exercise

Variable (pg/ml)	Western Diet + Exercise	Western Diet	Daniel Fast Diet + Exercise	Daniel Fast Diet
IL-4	37±9.6	25.9±7.8	36.3±9.6	27.1±9.6
IL-1β	174.5±59.5	120.9±55.1	31.4±59.5	72.3±65.2
IL-10	126.5±36.7	95.5±34	40.4±34	63.4±34
TNF-α	14.3±5.1	13.8±4.1	14.6±3.6	12.6±4.1

Values are mean±*SEM*.
No differences of statistical significance were noted ($p > 0.05$).

Table 3. Anthropometric data of male rats assigned to two different diets with and without exercise

	Western Diet + Exercise	Western Diet	Daniel Fast Diet + Exercise	Daniel Fast Diet
Body Mass (g) *Pre Intervention*	186.5±3.3	187.0±4.5	192.6±2.7	185±4.8
Body Mass (g) *Post Intervention*	516.8±10.7	571.1±14.7	478.7±11.3	496.8±13.5
Fat Mass (g) *Post Intervention*	161.6±8.0	195.5±8.4	100.73±7.4	124.45±9.8
Lean Mass (g) *Post Intervention*	366.0±9.2	386.8±6.7	391.4±8.8	376.5±7.8
% Fat *Post Intervention*	30.6±1.3	33.5±1.0	20.3±1.3	24.6±1.4

Values are mean±*SEM*.
A group effect noted for body mass ($p < 0.0001$).
A time effect noted for body mass ($p < 0.0001$).
A group by time interaction effect noted for body mass ($p < 0.0001$).
A group effect noted for fat mass ($p < 0.0001$).
A group effect noted for % fat ($p < 0.0001$).
No other statistically significant effects noted ($p > 0.05$).

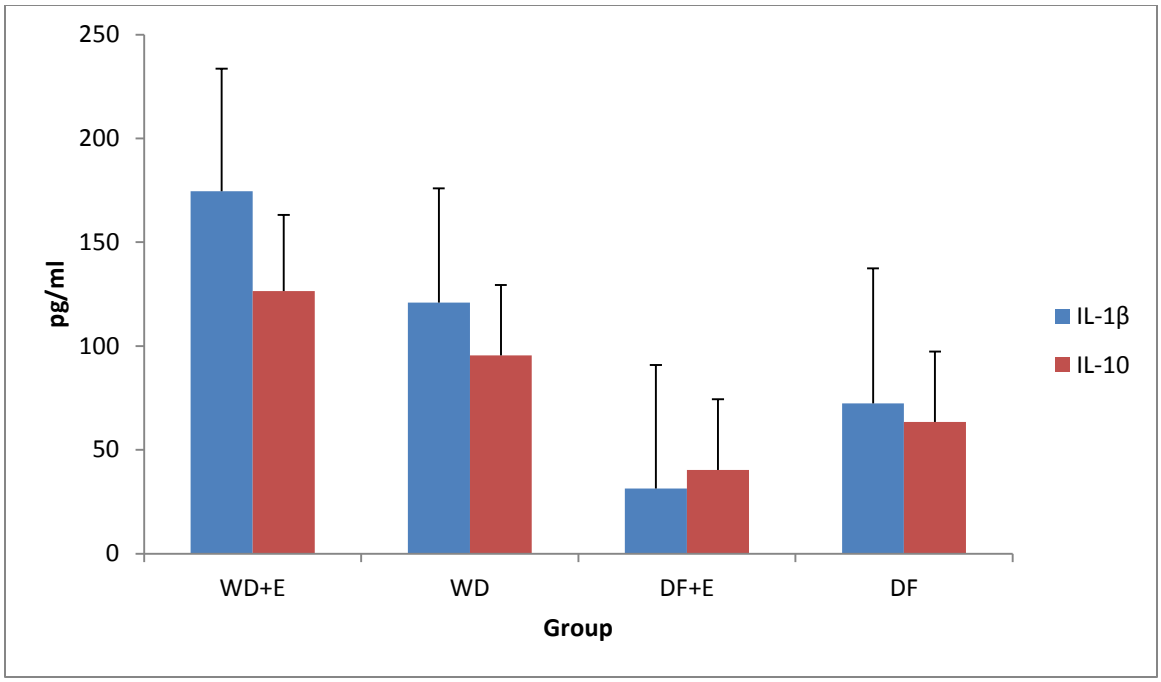

Figure 1. IL-10 and IL-1β mean concentrations between groups.

APPENDIX B- EXTENDED LITERATURE REVIEW

Overview of inflammation

Inflammation is classically defined as the body's natural response in dealing with injury. It plays a vital role in initiating and aiding in the healing process and acting as a crucial component of tissue repair (Hotamisligil, 2006; Punchard et al., 2004). More modern definitions of inflammation describe it as "the succession of changes which occur in a living tissue when it is injured provided that the injury is not of such a degree as to at once destroy its structure and vitality" (Punchard et al., 2004) and "the reaction to injury of the living microcirculation and related tissues" (Punchard et al., 2004; Spector & Willoughby, 1963). Physically, inflammation is characterized by five distinct signs: redness, swelling, heat, pain and loss of function (Allison et al., 1999; G. S. Hotamisligil, 2006; Punchard et al., 2004). These inflammatory characteristics are the immediate result of tissue damage and/or some forms of infection that occur in the body.

While short-term inflammation is both beneficial and necessary to the human body (Hotamisligil, 2006; Punchard et al., 2004), long-term inflammation is a main culprit of human disease (Cordain et al., 2005; Lakhdar et al., 2013; D. Thompson et al., 2010). Chronic levels of inflammation have proven to create major health concerns and produce adverse effects that underlie multiple diseases of the Western civilization (Cordain et al., 2005); diseases such as diabetes (Biondi-Zoccai et al., 2003; Dandona, Aljada, & Bandyopadhyay, 2004; Evans et al., 2002; Giannopoulou et al., 2005; Hotamisligil et al., 1995; Pradhan et al., 2001; Wellen & Hotamisligil, 2005), cardiovascular disease (Berg & Scherer, 2005; Bloomer et al., 2010; Burgmaier et al., 2010; Hilfiker-Kleiner, Landmesser, & Drexler, 2006), age-related diseases (Chung et al.,

2009; Hsiao et al., 2009; Rieu et al., 2009; Spaulding et al., 1997), cancer (Colotta et al., 2009; Deans & Wigmore, 2005; Gleeson, 2013; Komninou et al., 2006; Mantovani, Allavena, Sica, & Balkwill, 2008), cachexia (Deans & Wigmore, 2005; Sarraf et al., 1997; Tracey et al., 1988), and sarcopenia (Hsiao et al., 2009; G. L. Jensen, 2008; Rieu et al., 2009) are high on the rise and quickly becoming the main focus of world-wide attention in the research and medical fields. Due to the relation of inflammation and disease-related complications, it has been referred to as "the cornerstone of pathology" (Punchard et al., 2004). Undoubtedly, the volume and specificity of future research concerning inflammation will continually increase.

Health Implications of Inflammation

The Western diet, sometimes referred to as "The Standard American Diet" (SAD) (Grotto & Zied, 2010), has been associated with the development and progression of chronic low-grade inflammation ultimately resulting in the pathogenesis of specific diseases (Biondi-Zoccai et al., 2003; Bulló et al., 2003; Cordain et al., 2005; Galland, 2010; Gregersen, Samocha-Bonet, Heilbronn, & Campbell, 2012; Grotto & Zied, 2010; Vieira et al., 2009). Western dieting is characterized by a caloric intake rich in saturated fats, dense in refined carbohydrates, and high in sodium and cholesterol resulting in hypertension, hyperglycemia, dyslipidemia and a myriad of other symptoms (Burgmaier et al., 2010; Cordain et al., 2005; Galland, 2010; Gregersen et al., 2012; Grotto & Zied, 2010; Hsiao et al., 2009; Vieira et al., 2009). Increased visceral adipose tissue is the most notable physical manifestation of the Western diet (Hotamisligil, 2006). This accumulation of fatty tissue occurs primarily around the abdominal region and is one of the primary sites for the occurrence of inflammation (Dandona et al., 2001; Galland,

2010). Aside from the previously mentioned risk factors, chronic illnesses and diseases attributable to the Western dieting pattern have dramatically increased in the United States over the last few decades. For example, the CDC reported that heart disease was the leading cause of mortality in the United States for the year of 2012 at almost 600,000 deaths. A 2012 update on cardiovascular and metabolic disease estimates that 76 million Americans have hypertension and 25 million have type 2 diabetes (Writing Group Members et al., 2012).

Past studies have demonstrated that Dietary Restriction (DR), a dietary model that involves the removal of one or more components of dietary intake, could be a potential solution in ameliorating diet-induced impairments in health (R. Bloomer et al., 2010; Dandona et al., 2001; Koubova & Guarente, 2003; Spaulding et al., 1997). Various models of DR exist with a concerted effort to reduce the incidence of obesity and to lower chronic inflammation levels through the manipulation of dietary intake (Dandona et al., 2001; Dandona, Aljada, & Bandyopadhyay, 2004; Galland, 2010). Over varying time periods, DR has been effective in lowering blood pressure, blood glucose, cholesterol and C-reactive protein (CRP) levels (R. Bloomer et al., 2010; Walford, Harris, & Gunion, 1992). These desirable outcomes of DR have made it a multi-faceted tool in alleviating risk factors for a variety of ailments and diseases.

Measuring inflammation

Various tissues throughout the body are commonly targeted for measuring levels of inflammation. These include: the liver (Tordjman & Guerre-Millo, 2008), cardiac muscle (Burgmaier et al., 2010; Hilfiker-Kleiner et al., 2006), skeletal muscle (Montes et al., 2005), adipose tissue (Kern, Ranganathan, Li, Wood, & Ranganathan, 2001;

Tordjman & Guerre-Millo, 2008), and blood (Bloomer et al., 2010). It would be beneficial to analyze inflammation via multiple methods, as well as within various body tissues if results from blood warrant further investigation. Continued research in this domain is imperative in order to obtain a better grasp of the effects of inflammation both locally as well as systemically. For example, tumor necrosis factor-α (TNF-α), the first molecular link between obesity and inflammation, is produced in excess in adipose and skeletal tissues of obese humans (Hotamisligil et al., 1995; Hotamisligil, 1993; Kern et al., 1995; Saghizadeh et al., 1996; Sethi & Hotamisligil, 1999). As a result, measuring TNF-α concentrations in multiple sites throughout the body have shown to be highly beneficial in providing a more precise understanding of the nature of TNF-α and its relation to inflammation.

Current methods of measuring inflammation include: Biochemical measures, reverse transcription polymerase chain reaction, and protein expression, among others. Cytokines are soluble factors that mediate acute and chronic inflammatory responses within the immune system and between the immune system and other organs (Taniguchi, 1995). Understanding aspects of both pro- and anti-inflammatory cytokines is vital in order to gain a better grasp on the physiological processes that occur in the body. A wide range of biochemical assays are available for measuring human and animal cytokines (Burgmaier et al., 2010; Fichorova et al., 2008; Kern et al., 2001; Leng et al., 2008). Modern research studies aim to examine high levels of chronically circulating pro-inflammatory cytokines via assays, with data providing some understanding to their potential link to disease. Tumor-necrosis-factor-a, Interleukin-6 (IL-6), Interleukin-10, and Interleukin-1B (IL-1β) are among the most highly examined inflammatory cytokines

in relation to disease (Akira, Taga, & Kishimoto, 1993; Chang et al., 2013; Lakhdar et al., 2013; Lyke et al., 2004; Strohacker et al., 2013; Thompson et al., 2010).

Importance of inflammation in research

Because of the link between inflammation, disease, aging and age-related diseases, the continued study of inflammation is critical. Past studies have primarily addressed: cardiovascular disease (Burgmaier et al., 2010; Hilfiker-Kleiner et al., 2006) , diabetes (Evans et al., 2002; Wellen & Hotamisligil, 2005), cancer (Colotta et al., 2009; Mantovani, Allavena, Sica, & Balkwill, 2008), cachexia (Deans & Wigmore, 2005; Sarraf et al., 1997; Tracey et al., 1988), and sarcopenia (Jensen, 2008; Rieu et al., 2009) as they are all attributable to chronic inflammatory levels. Further examination of these diseases as they relate to inflammation is imperative for future success in preventative and treatment techniques. These studies will continue to progress into models of higher specificity over time in order to better comprehend the physiological pathways of inflammation.

Over recent decades there have been numerous studies centered on defining the role of inflammation in aging, age-related diseases, disability and frailty (Chung et al., 2009; Hsiao et al., 2009; Jensen, 2008; Leng et al., 2008; Malloy et al., 2006; Miller et al., 2005; Orentreich, Matias, DeFelice, & Zimmerman, 1993; Richie et al., 1994; Rieu et al., 2009; Weindruch & Sohal, 1997). Tumor necrosis factor-α, IL-1β, and IL-6 are the primary pro-inflammatory cytokines that have shown to increase in the sera of aged humans and mice (Chung et al., 2009; Rieu et al., 2009). Aged individuals tend to have higher levels of TNF-α, IL-1β, and IL-6 as compared to younger individuals (Chung et al., 2009). Chronic caloric restriction in aged individuals has shown to significantly

lower TNF-α, IL-1β, and IL-6 resulting in comparable levels to those of the younger individuals (Chung et al., 2009; Rieu et al., 2009).

Generally, inflammatory levels are affected differently by differing dietary patterns. Although numerous studies have been conducted utilizing various forms of DR concerning inflammation in varying tissues, none have investigated the effects of a completely purified diet. Dietary intake does appear to play a large role in regulating chronic low-grade inflammation over time (Grimble, 1992; Grotto & Zied, 2010).

Dietary intake and inflammation

Several previous studies have evaluated the impact of dietary manipulation on measures of inflammation (Dandona et al., 2001; Grandison, Piper, & Partridge, 2009; Grimble, 1992; Hasek et al., 2010; Holloszy & Fontana, 2007; Mager et al., 2006). These studies have found that dietary intake highly impacts inflammatory levels, either negatively or positively, depending on the type of diet assessed (Galland, 2010). Both the alteration in macro- and micronutrients is common to assess changes over time in various measures of inflammation.

Macronutrient manipulation includes regulating the levels and types of carbohydrate, fat or protein consumed in a daily diet (Wood, Volek, Davis, Dell'Ova, & Fernandez, 2006). This dieting strategy involves restricting one of the three macronutrients and increasing intake of one or two of the remaining nutrients. Because macronutrients constitute the majority of any diet, they generally produce significant changes in inflammation, more so than alterations in micronutrient intake.

Micronutrient manipulation may be described as modifying selected small nutrients, as well as nutrient supplements, in a diet. For example, calcium, vitamins D, E

and C, methylsulfonylmethane (MSM), conjugated linoleic acid (CLA) and other anti-inflammatory agents are among the most studied nutrients in research. However, studies regarding micronutrient manipulation are scarce as compared with macronutrient manipulation, with results dependent upon the type and magnitude of micronutrient differentiated in the diet (Grimble, 1992).

Dietary Modification Described

Multiple types of dietary manipulation exist including caloric restriction (CR), alternate day fasting (ADF), and DR. Dietary restriction may include the elimination in protein, as well as single amino acids such as methionine. Another model of DR is vegan dieting—which eliminates all animal products. An example of a vegan diet approach is the Daniel Fast, a dietary plan that involves consuming only plant-based foods but also calls for the elimination of all processed foods, refined flour, caffeine, flavorings, additives, and preservatives.

Each of the above dietary plans may result in favorable changes in inflammatory markers over time. However, long-term compliance to certain plans can be low, suggesting that benefits may be minimal. This is particularly true for plans in which calories are restricted.

Caloric restriction (CR) involves a specific reduction in calories, usually 20-40 % of daily intake, with little to no attention paid to which components of the diet are altered. Multiple studies have been conducted concerning CR in order to analyze various measures throughout the body and examine the role it plays in preventing disease (Chung et al., 2009; Harvey, Lashinger, Otto, Nunez, & Hursting, 2013; Koubova & Guarente, 2003; Mager et al., 2006; Spaulding et al., 1997).

Similar to CR, alternate day fasting (ADF) involves a reduction in calorie intake. This is typically done in 24-hour cycles in which food is consumed ad libitum for one day and then restricted totally or significantly (e.g., 500 calories) the next. The cycle repeats itself indefinitely. This form of dietary modification has been studied for its effects on chronic disease prevention in both humans and animals (Halberg et al., 2005; Heilbronn, Smith, Martin, Anton, & Ravussin, 2005; Johnson et al., 2007; Varady & Hellerstein, 2007).

Related to DR, past studies have examined the life-long DR of methionine in flies, rats and mice (Grandison et al., 2009; Hasek et al., 2010; Komninou et al., 2006; Malloy et al., 2006; McCarty et al., 2009; R. A. Miller et al., 2005; Orentreich, Matias, DeFelice, & Zimmerman, 1993; Perrone et al., 2008; Perrone et al., 2012; Plaisance et al., 2010; Plaisance et al., 2011; Richie Jr et al., 2004; Richie et al., 1994; Sun, Sadighi Akha, Miller, & Harper, 2009; Zimmerman, Malloy, Krajcik, & Orentreich, 2003). Results include: Increased energy expenditure, reduced plasma lipids, limited fat accretion, enhanced insulin sensitivity, extended median and maximum lifespan and increased weight-specific food consumption. The first clinical evaluation of dietary methionine restriction in humans yielded enhanced carbohydrate to fat oxidation and reduced hepatic and circulating triglycerides (McCarty et al., 2009; Plaisance et al., 2011).

Dietary protein restriction simply involves reducing overall protein intake. While otherwise healthy individuals appear to have no negative outcomes when consuming protein in high quantities, patients with chronic kidney and liver disease are often administered a diet that is low or absent in protein because of their body's inability to properly break it down. Previous studies have investigated the health benefits of

restricting dietary protein consumption. To their findings, the most important of these is a reduction in overall inflammatory levels (Choi et al., 2004; Grimble, 1992; Kaysen et al., 2001).

Aside from restriction of amino acids or whole protein, DR models such as vegan diets have been investigated (Choi et al., 2004; Craig, 2010; Kaysen et al., 2001). Findings in these studies indicate significantly healthier immune systems, lowered inflammatory levels and reduction in chronic disease rate. Because vegan diets are so varied, a mixed efficiency level exists for these findings depending on the exact style of vegan diet implemented. Many of these differing vegetarian diets are discussed in detail by Craig (Craig, 2010).

While vegan dieting allows the inclusion of processed foods, the Daniel Fast is a totally pure diet that excludes them. The concept of the Daniel Fast stems from the book of Daniel (10:2-3) in the Bible and implicates a strictly natural diet devoid of animal products, processed foods, artificial sweeteners, etc. Notable differences have been recorded concerning inflammatory levels in our lab while partaking in a Daniel Fast diet (Alleman et al., 2013; Bloomer et al., 2010).

Dietary modification – impact on inflammation

Multiple studies concerning the impact of CR on levels of inflammation have been conducted. The pro-inflammatory markers TNF-α, IL-1β and IL-6 generally increase in serum concentration as humans age (Chung et al., 2009; Rieu et al., 2009). The production of these cytokines is reduced by CR (Holloszy & Fontana, 2007; Spaulding et al., 1997), which is related to increased longevity. Results have also indicated that CR markedly reduces the incidence of malignancies (Weindruch & Sohal, 1997), systolic and

diastolic blood pressure, heart rate and body weight (Herlihy, Stacy, & Bertrand, 1992; Mager et al., 2006). The anti-inflammatory properties of CR make CR a viable dieting strategy in the attempt to reduce inflammatory-related disease and aging.

Studies investigating ADF have published mixed results. Subjects in the Halberg *et al.* study participated in three weeks of ADF. The results from this study indicated no significant reductions in TNF-α or IL-6 levels (Halberg et al., 2005). On the contrary, subjects in the Johnson study maintained an 8-week dietary ADF intervention. The results from this study suggest that TNF-α levels were strongly reduced. This can most likely be described due to the change in duration of dietary intervention between the two studies.

Although methionine restriction has shown many favorable outcomes concerning a variety of health parameters, to our knowledge there are minimal studies focused on the role of methionine restriction and reductions in inflammatory markers. Studies restricting methionine have reported a down-regulation of inflammation associated genes (McCarty et al., 2009; Perrone et al., 2012).

Protein restriction studies have noted significant reductions in pro-inflammatory markers (Choi et al., 2004; Grimble, 1992; Kaysen et al., 2001). The 1982 study by Keenan et al. gave the earliest indication that cytokine production is suppressed by a reduction in dietary protein intake (Keenan et al., 1982). Studies have since related high protein intake as producing inflammatory effects (H. K. Choi, Atkinson, Karlson, Willett, & Curhan, 2004; Grimble, 1992; Kaysen et al., 2001; Watanabe et al., 2005). Most of these studies focused on TNF-α and less on other pro-inflammatory cytokines (Grimble, 1992; Jennings & Elia, 1992; Keenan et al., 1982). Research on other inflammatory

markers of interest concerning protein restriction is relatively scarce, indicating the need for future research. C-reactive protein levels are the primary measurement for the studies that suggest a relationship between inflammation and protein intake. Current studies have labeled high circulating levels of CRP as an independent risk factor for cardiovascular disease (Lagrand et al., 1999; Lucas-Luciardi et al., 2012).

Vegan dieting has been investigated numerous times in regard to altering pro-inflammatory levels (Craig, 2010; Kaysen et al., 2001; Vlassara et al., 2002). However, because vegan diets also allow the consumption of refined carbohydrates and processed food, the specifications for the diet are vital in order to understanding the magnitude of impact it can have on inflammation. Vlassar*a et al. n*oted a significant reduction of 20 % in the pro-inflammatory cytokine TNF-α level following six weeks of vegan dieting (Vlassara et al., 2002). Other studies have produced similar results indicating a reduction in inflammatory marker levels in equivalence to the stringency of the dietary plan (Adam et al., 2003; Craig, 2010; Kaysen et al., 2001).

The Daniel Fast, devoid of refined carbohydrates and processed food, has been shown to reduce circulating CRP levels and lower white blood cell concentration (Alleman et al., 2013; Bloomer et al., 2010). Studies utilizing the vegan diet and Daniel Fast have only presently included measures in blood and would benefit from more advanced measures of the organs and tissues. Tissue samples may provide expertise on the local effects of inflammatory markers and gain insight into the long-term effects of inflammation.

Exercise and Inflammation

Another approach to reducing pro-inflammatory marker levels involves utilizing both exercise and diet. Exercise has been shown to provide protection against many diseases such as type 2 diabetes (Blair, Cheng, & Holder, 2001) and heart disease (Taylor et al., 2004). In a study by Thompson et al. (2010), 41 sedentary men were subjected to a 6-month exercise intervention. Throughout the study, inflammatory markers were monitored and recorded revealing that acute exercise immediately aids in reducing inflammatory levels and may be maintained chronically. Upon completion, a two-week detraining period was administered noting that the reduction in IL-6 was lost (Thompson et al., 2010).

Few studies have utilized diet, exercise and combination groups. Due to the high variability of diet plans and workout regimens, results vary in the few studies that have been conducted. Lakhdar et al. conducted a 6-month aerobic exercise+diet study that resulted in reductions in inflammatory markers in both the diet and diet+exercise groups. The exercise only group, however, showed no significant reductions in inflammatory levels (Lakhdar et al., 2013). More research is needed in this area combining exercise and diet in future endeavors as few studies have investigated the two in combination.

Conclusion

Both activity level and dietary intake have been shown to impact the chronic inflammatory status of an individual. The Daniel Fast, a stringent vegan diet, has demonstrated beneficial effects concerning human health and appears to favorably reduce inflammation. This is presumably due to the composition of the Daniel Fast (high concentration of low-glycemic carbohydrate and polyunsaturated fatty acids) as

compared to the Western diet (high concentration of saturated fats, refined sugars, and processed nutrients). Comparing the Daniel Fast and Western diet, coupled with sedentary or active lifestyle habits, may provide further understanding in regards to the current knowledge on inflammation. If favorable results are found in blood samples obtained from animals, future research may be conducted in additional animal tissues, in addition to larger-scale controlled human studies to test the impact of the Daniel Fast dietary regimen on inflammatory status.

APPENDIX C – IACUC APPROVAL

IACUC PROTOCOL ACTION FORM

To:	Rick Bloomer
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	9-25-13

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. Dietary modification in rats (0734)

☒ Your proposal is approved for the following period:

From: September 25, 2013 To: September 24, 2016

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Revision Memorandum dated _____ for the following period:

From: _____ To: _____

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Revision Memorandum dated _____.