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Leptin effects on bone metabolism

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Abstract:

Scales from adult zebrafish, *Danio rerio,* are an established model for studying osteoblast and osteoclast activity. In this study, scales in a cell culture were used to determine if the hormone leptin has an effect on activity of these bone remodeling scales. Alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities of zebrafish scales were measured and compared with and without leptin treatment. There was no statistically significant difference between leptin treatments in terms of ALP and TRAP activity. Differences between TRAP values between days 1 and 2 did look promising although they were not statistically significant. This study could provide an insight into any effect that leptin may have on bone metabolism considering the differences in TRAP activity as previously mentioned. A viable scale assay model, however, was established which can contribute to the understanding of the molecular role of leptin in bone metabolism.

Introduction:

Seen as a "master hormone" (Takeda, et al. 2002), leptin regulates appetite and metabolic rate, as well as reproduction, immune function, and bone growth/resorption (Londraville, et al. 2017). Bone growth and resorption are of interest in terms of how to treat osteodegenerative diseases (e.g., osteoporosis), and leptin is of research interest due to its antiosteogenic and anorexigenic effects that act independently of each other. Antiosteogenic and anorexigenic effects are associated with the sympathetic and central nervous systems, respectively (Takeda, et al. 2002; Philbrick, et al. 2017). Because it must cross the blood-brain barrier, leptin has limited entry into the central nervous system (Philbrick, et al. 2017). Normal bone growth in mice requires leptin, and without signaling, skeletal abnormalities - including decreased bone length and total bone-mass can occur. These abnormalities can be reversed by administering the

hormone, but too much administered directly into the hypothalamus can activate leptin's antiosteogenic effects (Philbrick, et al. 2017). Leptin does not affect the total number of osteoclasts, but rather their activity (Philbrick, et al. 2017; Pasqualetti, et al. 2012).

In mammals, leptin is synthesized by adipose tissue and travels to the hypothalamus across the blood-brain barrier where it binds to the leptin receptor and regulates appetite and lipid mobilization for metabolism; in bony fish, such as zebrafish, it is primarily expressed in the liver (Londraville, et al. 2017). Zebrafish express both LEPA and B as well as their subtypes. They are effective models when studying the possible effects of leptin on humans since there is some evolutionary conservation of tertiary protein structures after bony fish diverged from humans. Leptin binds to its receptor in a zebrafish hypothalamus in a similar fashion to a human's due to the conservation of the protein folding (Londraville, et al. 2017).

Zebrafish scales, like human bone, have osteoblasts and osteoclasts (bone-forming and resorptive cells, respectively), making zebrafish scales ideal when studying leptin's effects on osteogenesis. Osteoblasts have been known to play a role in bone-formation as well as regulating osteoclast maturation, which then perform bone resorption (Tanaka, et al. 2005). They also have all organs for metabolic activity present in humans (Carnovali, et al. 2018). Zebrafish elasmoid scales form internal circuli when the scales are forming new collagen fibers into the bone matrix, as can be visualized using staining techniques (Pasqualetti, et al. 2012 A). Osteoblasts are present on both the episquamal and hyposquamal surfacess of the scale. On the episquamal surface, they deposit a mineralized tissue layer organized in circuli, while the hyposquamal side deposits collagen that makes up the basal plate of the scale (Pasqualetti, et al. 2012 A). The episquamal has two areas, the central and lateral areas, where the osteoblasts are arranged differently. Osteoblasts in the central area are distributed randomly and organized in cords where

ring mineralization is more consistent. In the lateral area, osteoblasts are organized in a well-defined pattern along the circuli, and more circuli form in a cranial-caudal pattern, adding to the scale surface of the zebrafish (Pasqualetti, et al. 2012 A).

There also seems to be a connection between obesity and bone health as shown by Carnovali, et al. (2018). They found that in zebrafish fed a high-fat diet, there was decreased alkaline phosphatase (ALP) activity and increased tartrate-resistant acid phosphatase (TRAP) activity. ALP is characteristic of osteoblast (bone cell formation) activity and thus can be used to quantitatively measure the osteoblastic activity. TRAP assays indicate osteoclast (bone cell resorption) activity and is used as a quantitative measure for osteoclast activity. Carnovali, et al. (2018) found that increased leptin along with a decrease in adiponectin secretion were associated with bone resorption in obese fish, explaining the increased TRAP activity. With its similarities in bone metabolism to humans, zebrafish can be used to understand if leptin is related to bone metabolism and spur future research into the hormone's role in bone diseases such as osteoporosis. Thus, our main objective is to determine if leptin has a significant effect on bone metabolism in terms of ALP and TRAP activity. We believe that leptin may have some effect on osteoclast activity after evidence seen in mice, where normal bone growth requires leptin (Philbrick, et al. 2017).

Methods and Materials

Animals

Adult zebrafish of AB stock (ZIRC) from the Londraville lab colony were used. Zebrafish used for scale removal were anesthetized by being placed in 0.01% MS222 until they lost equilibrium. Total wet weight (0.351 g ± 0.106) and length (34.6 mm ± 2.52) of each fish (n

= 5) were measured. Scale removal was performed under a dissecting microscope using a wetted stage and watchmaker forceps to remove scales from just behind the operculum. No more than 3 scales from each side of the fish were removed at a time. Removed scales were placed in phosphate-buffered saline (PBS) before being placed in cell culture medium for incubation.

Figure 1: *D. rerio* (zebrafish). White arrow shows location of the operculum. Red arrow shows the area where a scale was extracted from each side of the fish. (source: https://www.britannica.com/animal/zebra-danio)

Culture Medium Preparation

Culture medium was prepared to determine if it was possible to keep an isolated zebrafish scale alive and growing over a 48-hour period. Culture medium was prepared using L-15 Leibovitz Medium with a pH indicator, Phosphate Buffer Saline (PBS), Penicillin-Streptomycin mixture, and Fetal Bovine Serum as described by Pasqualetti, et al., (2012). Preparation of the mixture was performed in a sterile cell culture hood. 500 mL total of culture medium was made using 450 mL L-15, 50 mL PBS, and 2% of antibiotics relative to the total volume of medium. Since the Penicillin-Streptomycin mixture was at 100x concentration, 5 mL was added to the solution. From a 500 mL bottle of L-15, 55 mL was removed and discarded. 50 mL of PBS was added to the remaining L-15, then 5 mL of antibiotic mixture, and the entire solution was mixed.

The medium was supplemented with 10% fetal bovine serum. Aliquots of 5 mL were placed in sterile 7 mL vials and refrigerated.

Incubation and Assay Troubleshoot

Initial trials were contaminated by bacterial growth as was indicated by a color change of the culture medium from pink to yellow. Sterilization of scales was subsequently performed after removal to reduce contamination. The scales were removed from the fish, immersed in 70% ethanol for 5 seconds, then placed in PBS for transportation to the cell culture hood for placement into medium. Originally, wells were used for incubation, but showed signs of leakage into neighboring wells. Thereafter, individual 200 μL centrifuge tubes were instead used for individual scale incubation. Scales were originally homogenized using a Sonicator, and thereafter with a Bullet Blender (West Advance, Troy, NY) using 1.0 mm zirconium oxide beads.

Incubation

Scales (n=6) freshly removed from the fish (no culture) were assayed for ALP and TRAP activity for comparison to scales in culture. Remaining scales (n=12) were placed in centrifuge tubes with 100 μL of culture medium. Scales were incubated in individual centrifuge tubes in a shaking incubator at 28℃ for 48 hours. After 24 hours, 6 scales were removed from incubation and assayed for ALP and TRAP activity. The remaining 6 scales received new media and were tested under the assays after another 24 hours, for 48 hours total incubation.

Performance of Assays and Proper Incubation

To remove the incubated scales from the centrifuge tubes, they were centrifuged for 45 seconds at 1,000 rpm and the medium was removed using a micropipette. The ALP assay was

performed as described by Persson, et al. (1995) and the TRAP assay was performed as described by Suzuki, et al. (2009). Modifications were made to the ALP assay as follows: the scales were homogenized, 180 µL of buffer and 0.674 M of pNPP, and no stop solution was added after the 2-hour incubation time. Modifications made to the TRAP assay were as follows: 180 µL of buffer and 0.674 M of pNPP were used. Scales that were tested for Day 0 ALP and TRAP results were immediately placed in assay buffers after sterilization. Once in assay buffers, the scales were homogenized in the bead beater twice at 5-minute intervals, for a total of 10-minute homogenization, then incubated in a digital incubator for 2 hours at 28℃. Controls received the same incubation treatment. Samples were then placed in a microplate reader wells and read using a at 405 nm. The process was repeated for samples incubated after 24 and 48 hours. ALP and TRAP net activity was processed through a one-way ANOVA test using Microsoft Excel.

On Day 0 of trials, no leptin was added to any samples, and 6 scales were tested to determine a baseline. On days 1 (24 hours) and 2 (48 hours), 0.5 µM recombinant zebrafish leptin A (Genscript) was added to 12 scales and 12 scales received no leptin. Fresh 15 µM aliquots of leptin was added to the day 2 scales when media was changed daily. These aliquots were pre-prepared and frozen when not in use.

Table 1: Treatments on scales per day and assay. X lep = no leptin treatment; lep = leptin treatment.

Day:	Day 0		Day 1 (24 hs)				Day 2 (48 hs)			
Assay:	ALP	TRAP	ALP		TRAP		ALP		TRAP	
Treatment:	X lep	X lep	X lep	lep	X lep	lep	X lep	lep	X lep	lep
# scales:	3	3	3	3	3	3	3	3	3	3

Results:

All fish were measured for length and weight and their mean and standard deviations calculated. The mean length was 34.56 mm ± 2.52 mm and the mean weight was 0.351 g ± 0.106 g (n = 18 fish). Table 2 shows the net background activity for all treatment groups over the period of the study. Table 3 provides the lengths and weights of all zebrafish individuals used, as well as the mean and standard deviation among the individuals. Net activity of ALP and TRAP was calculated using a one-way ANOVA, where net activity was calculated by subtracting the control from background activity. The ALP one-way ANOVA found a p-value of 0.729, as seen in Table 4, which is not statistically significant. TRAP one-way ANOVA results showed a p-value of 0.530, as seen in Table 5, which is also not statistically significant. In Fig. 2, outliers of ALP values are present. Furthermore, Fig. 2 shows no significant difference among the treatment groups. Fig. 3 shows similar results for TRAP activity with no significant difference among the treatment groups and outliers present.

Table 2: Net ALP and TRAP activity. Net = activity – background. Negative values were due to the control value being larger than the background activity.

	ALP X lep	**ALP lep**	**TRAP X lep**	**TRAP lep**
9/25/2019	0.279	0.352	0.052	0.011
	0.587	0.252	0.007	0.023
	0.55	0.928	0.023	0.025
9/26/2019	0.197	0.408	0.016	0.014
	0.128	0.612	0.079	0.005
	0.008	0.151	0.012	0.034
10/2/2019	0.426	0.473	0.006	0.007
	0.538	0.369	0.006	0.012
	0.653	0.233	0.015	0.01
10/3/2019	0.343	0.208	0.006	0.011
	0.259	0.2	0.016	0.022
	0.176	0.148	0.013	0.014
10/9/2019	0.489	0.311	0.01	0.06
	0.361	0.493	0.011	0.009
	0.55	0.482	0.011	0.01
10/10/2019	0.241	0.283	0.035	0.041
	0.184	0.15	0.019	0.04
	0.316	0.265	0.032	0.02
10/16/2019	0.895	0.628	-0.01	-0.011
	0.279	0.318	-0.001	0.048
	1.151	0.65	0.008	0.013
10/17/2019	0.652	0.683	0.014	0.037
	0.499	1.246	0.034	0.029
	0.776	1.796	0.038	0.024
10/23/2019	0.457	0.542	0.076	0.086
	0.475	0.559	0.068	0.088
	0.421	0.264	0.072	0.186
10/24/2019	0.155	0.224	0.027	0.025
	0.315	0.171	-0.004	0.004
	0.325	0.111	0.062	0.01

Table 3: Weight (g) and length (mm) of each fish used. Average weight and length were calculated as well as the standard deviation

Day	**weight (g)**	**length (mm)**
9/24/2019	0.284	32
	0.222	36
	0.289	31
	0.342	35
	0.195	35
10/1/2019	0.373	35
	0.245	33
	0.347	36
	0.266	32
	0.188	29
10/8/2019	0.582	38
	0.472	39
	0.259	33
	0.242	30
	0.489	36
10/15/2019	0.386	35
	0.359	34
	0.383	35
	0.312	33
	0.563	38
10/22/2019	0.416	39
	0.409	35
	0.296	34
	0.373	35
	0.482	36
Average	0.35096	34.56
SD	0.105707	2.5152336

Table 4: ANOVA single factor summary chart for ALP net activity. There is no effect of leptin treatment on ALP activity (p = 0.729).

Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	30	12.685	0.422833	0.059098		
Column 2	30	13.51	0.450333	0.128378		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011344	1	0.011344	0.121016	0.729195	4.006873
Within Groups	5.436795	58	0.093738			
Total	5.448139	59				

Table 5: ANOVA single factor summary chart for TRAP net activity. There is no effect of leptin treatment on TRAP activity (p = 0.530).

Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	30	0.753	0.0251	0.000616		
Column 2	30	0.907	0.030233	0.001367		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000395	1	0.000395	0.398634	0.530276	4.006873
Within Groups	0.05751	58	0.000992			
Total	0.057905	59				

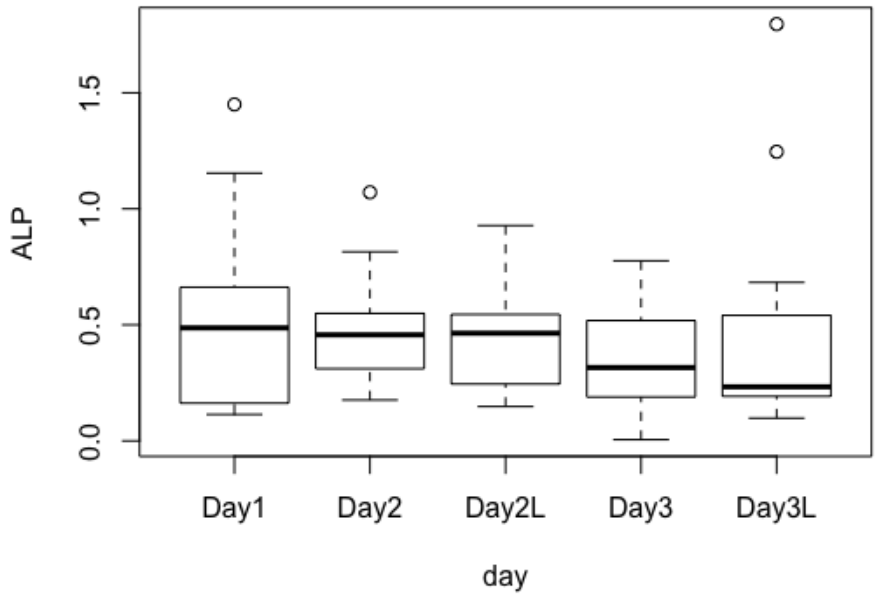

Figure 2: Box and whisker plot of ALP activity. Days 1, 2, and 3 received no leptin treatment. Days 2L and 3L received treatment.

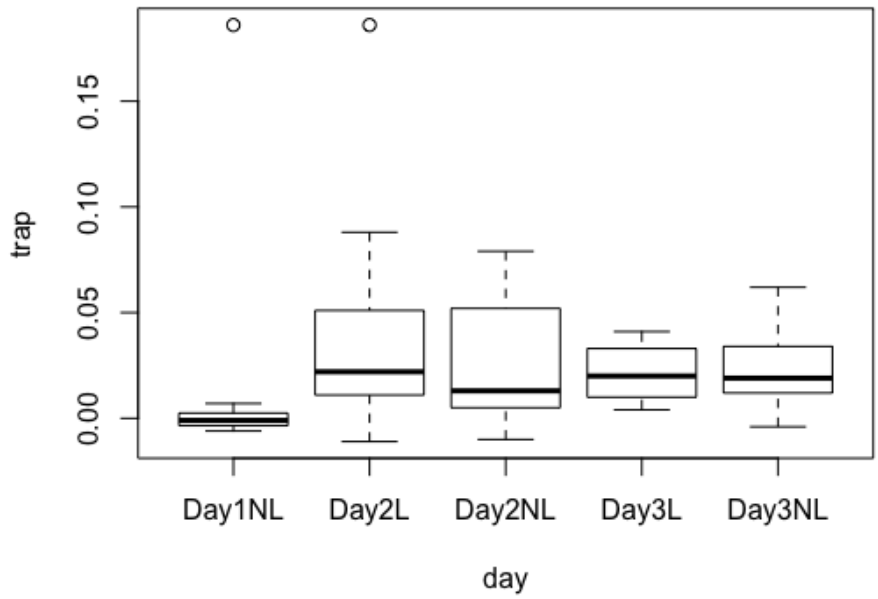

Figure 3: Box and whisker plot of TRAP activity. Days 1NL, 2NL, and 3NL received no leptin treatment. Days 2L and 3L received treatment.

Discussion:

Leptin's role in vertebrate osteogenesis is not fully understood. During this study, effects of leptin on osteoblast and osteoclast activity was observed and was found to have no significant impact on the activity of these bone cells. While there is a trend for TRAP activity to be higher in leptin-treated scales, it is not statistically significant. As such, it can be assumed that leptin does not have a significant effect on osteoblasts or osteoclasts based on this study.

Multiple issues were found while performing this study. Single scales were to be utilized for each value. However, due to the small nature of scales and their tendency to adhere to one another, there were instances where some values could have measured two scales instead of one. While the scales are visible to the naked eye, at times it was difficult to determine if a scale was present in individual centrifuge tubes because the scales' translucent appearance. At times a small amount of pigmentation could be seen on the scale, but not on every specimen. Furthermore, not all scales were similar in size, so some scales were smaller than others, possibly causing variability in results. Issues also arose when absorbance values of samples were found to be less than the control absorbance values. In these cases it was assumed that no scale was present in the sample.

In terms of scale size, there were difficulties in ensuring that all scales were of similar sizes. We found that there were both large and small scales within the sample pool. Attempts were made to determine the average weight of a scale but were unsuccessful due to the size of the scales. A large number of scales at one time (dozens) was needed to register at all on our most sensitive scale. Removing such a large amount of scales can be unsafe for the fish, so it is inappropriate to remove so many to find an average scale weight. Leptin was also to be added every day to the appropriate tubes at the same time, but this did not occur. At times leptin was

not added until a few hours before activity was to be measured. This added variability to leptin incubation times. Lastly, there were some instances where the homogenized scales were incubated for 2 hours at temperatures greater than 28℃. Enzyme activity could have been affected by this change in temperature.

Carnovali, et al. (2018) performed a zebrafish scale assay to understand how a high-fat diet affects bone metabolism. They found that scales of fish on the high-fat diet had increased leptin and showed increased TRAP activity and decreased ALP activity on their scales, causing an "osteoporosis-like phenotype" (Carnovali, et al. 2018). These results are consistent with the increased (but not significant) TRAP activity seen in this study. The research done by Carnovali, et al. (2018) shows that leptin does have an effect on bone metabolism when a high-fat diet is taken into consideration. High fat diet changes many variables at once, and another factor may be what is affecting scale dynamics (such as insulin). My data provides insight to the possibility of leptin alone driving the effect of diet on scale growth.

Another study by Frøiland, et al. (2012) studied Arctic charr to determine if leptin plays a role in adiposity. Their treatment groups included Arctic charr that were fed in excess from March to November and that were food-deprived from March to June, then fed in excess again from July to November. They found that leptin increased during the winter months (normal fasting time) while the fish lost body fat and were anorexic. Since leptin activity decreased, it may also have an effect on scale growth and bone metabolism. Our scale assay can provide insight into how bone structure could change in Arctic charr during this fasting time. Carnovali et al. (2018) has already demonstrated an effect of leptin on bone metabolism with a high fat diet, and our scale assay could be implemented into studies like Frøiland, et al. (2018) to understand any of leptin's effects on bone metabolism during fasting.

More research should be performed to determine if leptin truly does influence osteogenic pathways. Some values for TRAP had looked promising due to there being a difference in absorbance values between days 2 and 3 when leptin was added. If variability could be decreased, such as by microscopically calculating the area of each scale and expressing activity per unit area, it is possible that an effect could have been seen. This could be performed by microscopically taking a photograph of the scales and digitally scaling the sample. As previously stated, the weight of a single scale is difficult to measure due to its small size, and multiple scales would be required to find an average weight of a single scale. Removal of multiple scales from one zebrafish can cause injury to the individual. Thus, area of the scale is a more feasible measurement than weight. By determining the amount of activity per unit area, a more precise way of determining if leptin has a significant effect can be developed.

Leptin regulates many pathways including reproduction, immune function, and bone growth/resorption (Londraville, et al. 2017). Studies into how it affects osteogenesis can be continued and possibly used in understanding bone diseases such as osteoporosis. It is known that leptin does play a significant role in proper bone formation in mice, as shown by Philbrick, et al. (2017). However, whether it plays a significant role for zebrafish is still to be determined: very few studies exist researching the effects of leptin on bone metabolism in *D. rerio*, so this study has contributed to a hopefully growing area of research. If leptin truly does have a significant effect on bone cells, the master hormone can then be used in studies of how to treat bone degenerative diseases using zebrafish scales as a model.

Conclusion:

This research was performed to determine if leptin has a significant effect on osteoclasts and osteoblasts in a zebrafish scale assay model. ALP and TRAP activities were used as signals

for osteoblast and osteoclast activity, respectively. Scales that received leptin treatment showed no significant difference in ALP or TRAP activity as compared to scales that received no treatment. It is possible that with better control of individual activity, such as amount of activity per unit area of scale, a significant difference could be observed. We hope to have future studies that include trying to determine amount of activity per unit area to investigate more into leptin's effects on bone metabolism. However, this work established that the zebrafish scale assay model is a viable model to investigate leptin's effects on bone metabolism in fish.

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