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Analyzing Oxytocin Levels Using Liver Tissue from Fasting Mice

By

Mark Kamulegeya.

BSc. (Biochemistry, Botany)

Reg No: 18/U/14222/Ps

Supervisor: Dr. Julius Mulindwa

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SEPTEMBER, 2022

DECLARATION

DECLARATION

I **Kamulegeya Mark**, declare that the work compiled in this research dissertation entitled "**Analyzing Oxytocin Levels Using Liver Tissue from Fasting Mice**." has never been submitted to any institution in partial fulfillment of the requirement of any award and that it has been entirely my effort.

Signature: ..... Date: 18/Oct/2022

KAMULEGEYA MARK
18/U/14222/PS
BSc. Biological,
Department of biochemistry and sports science
School of Biosciences
College of Natural Sciences
Makerere University

APPROVAL

This research dissertation entitled "**Analyzing Oxytocin Levels Using Liver Tissue from Fasting Mice**" has been submitted with approval of my academic supervisor.

Supervisor

.....

Dr. JULIUS MULINDWA Date 18/10/2022

Lecturer

Department of Biochemistry and Sports Sciences

School of Biosciences

College of Natural Sciences

Makerere University

DEDICATION

I dedicate this work to my S.4 Biology students' class of 2022 of Jamuiyatul Tawheed Islamic Secondary School-Wakiso.

ACKNOWLEGEMENT

I would like to appreciate the Almighty God so much for the gift of life and for having enabled me to complete my project.

My heartfelt thanks go to my academic supervisor Dr. Julius Mulindwa for the guidance as I did my research work.

Special thanks to Mr. Lujjumba Ibra for the assistance he gave me as I did my research work.

ABSTRACT

Oxytocin is a central nervous system neuropeptide hormone, which is composed of nine amino acids. The synthesis of oxytocin begins in the hypothalamus, where the paraventricular nucleus and supra-optic neurons express high levels of oxytocin, which is released from the posterior pituitary gland. Whereas "Fasting" may be defined as voluntary abstinence from food and drink for specified, recurring periods of time, with the fasting periods typically ranging from 12 hours to three weeks in humans. Hippocrates recommended fasting during sickness and was probably one of the first advocates of fasting for medical purposes. There exists limited information showing a direct relationship between oxytocin synthesis and fasting.

In order to determine the change that occur in oxytocin levels during fasting, Lnpep a gene that is involved in its metabolism in the liver was considered. Its levels of expression were to be monitored under different conditions. Data was obtained from the NCBI Gene Expression Omnibus (GEO) database; two datasets were selected for this study, (accession numbers GSE137385 and GSE130127). From the different GSEs, extrapolation of the following dataset information was done: run, assay type, library layout, organism, platform, strain, tissue and treatment. From GSE130127, selected were 9 runs beginning SRR89371 which is represented as X followed by the last 3 digits from each run as shown from (table 1) through the analysis of the results. In the first group AL had 3 samples (X153, X154,X155) , in the second group IF16h3wks 3 samples were selected (X158,X159,X160), in the third group IF24h3wks 3 samples were selected (X163,X164,X165) and only 3 samples were selected from GSE137385, the 3 runs beginning with SRR10112 which also was represented as X followed by the last 3 digits from each run as shown in, thus the fourth group 21fast 3 samples were selected (X565,X566,X567). A total of 12 samples were selected for comparing the different time points i.e (fasting for 21 hour a one day fast 21fast, fasting for 16 hours for 3 weeks IF16h3wks, fasting for 24 hours for 3 weeks) IF24h3wks and the fed condition 0 hours of fasting AL.

The gene signatures exhibited by mice under ad libitum feeding (AL), mice that fasted for 21 hours(21fast), mice under intermitted fasting for 16 hours in 3 weeks (IF16h3wks) and mice under

intermitted fasting for 24 hours in 3 weeks (IF24h3wks) indicate differentiation in gene expression according to the PCA, the abnormality seen in the 21fast may be due to the presence of the different mouse strains in the this group this resulted in the genes with expression levels significantly higher in C57BL/6J mice than in BALB/cJ mice and distinct clustering of experimental groups using heat map analysis. Even though common genes that are differentially expressed by in all fasting groups and AL are present, it can be further observed that a large number of differentially expressed genes are unique to either the 21fast or the IF24h3wks regimen. This showed that the longer the fasting time of the day the more genes are significantly expressed. Fasting resulted as expected in marked weight loss with reduction of abdominal circumference, which was more pronounced in the groups which was fasted longer. On the other side, Pilot data suggest that longer -term oxytocin administration may support weight loss in obese patients. These findings provided evidence that fasting longer could be similar to longer-term oxytocin administration. Since a particular level of oxytocin is attained when an individual fasts thus, as one fasts longer this dose of oxytocin is attained, thus bring about a similar effect observed in fasting and oxytocin administration.

These results provide evidence that there is a direct relationship between fasting oxytocin synthesis levels in mice. It was noted that there is threshold oxytocin level which was observed in all fasting groups and this could be the required oxytocin level need to bring about the beneficial effects observed during fasting. However, this calls for further studies to monitor oxytocin synthesis using brain tissue as to understand which exact genes involved in its synthesis are up regulated and to what extent during different fasting conditions.

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CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Oxytocin is a central nervous system neuropeptide hormone, which is composed of nine amino acids. The synthesis of oxytocin begins in the hypothalamus, where the paraventricular nucleus and supra-optic neurons express high levels of oxytocin, which is released from the posterior pituitary gland (viero et al., 2010).well as, "Fasting" may be defined as voluntary abstinence from food and drink for specified, recurring periods of time, with the fasting periods typically ranging from 12 hours to three weeks in humans (valter et al., 2014). Hippocrates recommended fasting during sickness and was probably one of the first advocates of fasting for medical purposes (Yiren et al., 2022). In 1800s, Edward Dewey adopted a somewhat radical view of fasting and believed that virtually all disease stemmed from excessive eating (Matthew, 2019). “Fasting is without any doubt, the most effective biological method of treatment” wrote by Buchinger as he documented the beneficial effects of fasting in many human diseases during the 1900s.One of the most recent suggestions was by Valter Longo, in the 2000s, that, fasting selectively activates multiple “longevity programs” which may lead not only to an extended life span, but also to an extended health span. (Longo, et al., 2017; Anton, et al., 2018) . Long periods of fasting that were termed the "zero calorie diet" were used to treat morbid obesity and associated diseases. (Ditschuneit, et al., 1970; Runcie, et al., 1974; Françoise, et al.,2019). Obesity is associated with an increased risk of developing several cancers, such as breast cancer, colon cancer, ovarian cancer, endometrial cancer and thyroid cancer. (Bhaskaran, et al., 2014; Chan, et al., 2014). Fasting may also delay aging, a major risk factor for neurological diseases. (Longo, et al., 2014; Anton, et al., 2018). Having three or more meals a day has been related to prevalence of obesity, type 2 diabetes and a variety of disabling neurological disorders. (Word Health statistics, 2018; pringsheim, et al., 2014). A number of factors contribute to the development of cardiovascular diseases such as age,

gender, obesity, disorders of lipid metabolism, hyper tension, diabetes and poor diet among others. (Matyjaszczyk, et al., 2011).

1.2 Problem statement

There exists limited information showing a direct relationship between oxytocin synthesis and fasting. However, both Fasting and oxytocin have been observed to exert similar effects in regulation of bodily process which include; hunger inhibition, stimulation of endogenous opioids resulting in the expression of good feelings and emotions, decrease in blood pressure, remarkable weight loss, reduction in leptin levels and decrease in insulin levels. This leads to the hypothesis that oxytocin hormone levels raise during fasting, thus oxytocin exerting the beneficial effects observed during fasting. However, the raise in oxytocin levels may vary depending on type of fasting and duration of the fast.

1.3 Study objective

1.3.1 General objective

To analyze oxytocin synthesis in fasting mice using already published transcriptomic data from NCBI data base.

1.3.2 Specific objective

1. To identify already published transcriptomic data of fasting mice from NCBI data base.

2. To monitor the expression patterns of the Lnpep gene in both fasted and no fated state.

3. To determine whether oxytocin synthesis levels increase during fasting mice.

1.4 Justification

Since oxytocin is rapidly metabolized in the liver by secreted oxytocinase (leucyl/cystinyl aminopeptidase) genes with a gene symbol Lnpep and gene id number ENSMUSG00000023845, this will be used for this study analysis to determine its expression levels under different fasting and non fasting conditions in mice.Expression for oxytocinase synthesis will be used to determine the rate of breakdown of oxytocin in liver, study analysis will be done using mice expression data from which highl levels of this gene expressed will reflect increased oxytocin synthesis with in the mice while lower levels of the oxytocinase synthesis gene will imply lower oxytocin synthesis with in the mice.(Elkins et al.,2017).

1.5 Significance

Combination of both fasting and oxytocin administration could provide an effective therapy in re-sensitizing a wide range of resistant cancer cells(Novella, et al., 2018), but also administration of oxytocin may reduce side effects associated with chemotherapy without negatively impacting tumor cell killing, in cases were fasting is not feasible for all patients, especially the elderly or those exhibiting cachexia.(Levine, et al., 2014; Tinkum,et al., 2015:Ali, et al., 2018).

CHAPTER TWO: LITERATURE REVIEW

2.1 Fasting and oxytocin effect on hunger:

During an alternated day fasting and time restricted fasting diets, the perceived feeling of fullness increased during the study period relative to the baseline assessment, which may contribute to long term adherence to such a diet.(Cai, et al., 2019; Françoise, et al., 2019).In similar fashion, acute intranasal administration of oxytocin inhibits reward but also hungry driven food intake in obese Men.(Thienel, et al., 2016).Oxytocin has also been shown to inhibit feeding after intracerebroventricular injection conducted on a number of experiments in rodents. (Arletti, et al., 1989; Olson, et al., 1991). Oxytocin also exerts a potent acute inhibition of food intake in obese subjects which even surpasses the effect found in normal weight humans. (Thienel, et al., 2016). Oxytocin might furthermore restrain food intake by acting on downstream mediators of the leptin signal. (Blevins, et al., 2004). Leptin levels are also reported to have decreased during fasting a hormone predominantly made by adipocytes that inhibits hunger. (Mathew, 2019; Bartosz, et al., 2019; Yongin, et al.,2019; David, et al., 1997). In contrast, it has been reported that short fasting periods of two days, as well as alternate day fasting, are associated with the feeling of hunger. (Solianik, et al., 2018; Heilbronn, et al., 2005; Johnson, et al., 2007).

2.2 Fasting and oxytocin effect on endogenous opioids.

Fasting leads to continuous increase in emotional as well as physical wellbeing, this was evident across all groups of different fasting period length. This is an important component to increase compliance and has been reported in earlier studies based on daily mood ratings. (Runcie, et al., 1974; Michalsen, et al., 2016; Michalsen, et al., 2003; Françoise, et al., 2019). Endogenous opioids could also contribute to the wellbeing, as documented in a 10-day fasting trial in men. (Komoki, et al., 1990). However, oxytocin a peptide hormone and neuropeptide, its production is associated with good feelings and emotions. (Magon, et al., 2011). In addition to activating its own receptors and decreasing pain signals, oxytocin binds to opioid receptors and stimulates endogenous opioid release in the brain. (Reeta, et al., 2006; Breton, et al., 2008; Gimpi, et al., 2001). Oxytocin also stimulates cannabinoid

receptors and is known to relieve pain as well as induce a feeling of calm, and lower serum cortisol, stress, and anxiety. (Windle, et al., 1997).

2.3Fasting and oxytocin effect on blood pressure:

Benefits of fasting are improved cardiovascular risk factors, such as a decrease in blood pressure, improvement of lipid profile and insulin sensitivity and weight loss in obese and non-obese subjects. (Françoise, et al., 2019; Goldhamer, et al., 2002). Intracerebroventricularly injected Oxycontin decreased blood pressure, whereas inhibition of brain oxytocin synthesis by an antisense oligonucleotide increased blood pressure in rats. (Maier, et al., 1998). In primates, humans, and rats the administration of oxytocin is often associated with a decrease in blood pressure. (Petersson, et al., 1996; Petersson, et al., 1997; Gutkowska, et al., 2000).

2.4 Fasting and oxytocin effect on insulin levels:

A Number of diseases have been strongly associated with insulin resistance (IR) /type 2 diabetes mellitus (T2DM), obesity, and the metabolic syndrome which may eventually lead to complications such as Cirrhosis, Liver cancer, liver failure, or cardiovascular diseases. (Divella, et al., 2019; Sarwar, et al., 2018; Dharmalingam, et al., 2018). Type 2 diabetes is a common metabolic disorder in the world. It correlates with an increase in obesity rates and sedentary lifestyles. Limiting the development of diabetes prevents many diseases including cardiovascular diseases. (Arnason, et al., 2017). Diabetes induced by obesity is characterized by hyperglycemia, insulin resistance, and progressive beta cell failure. (Lesile, et al., 2016; van, et al., 2009). Fasting on alternate days for three weeks decreases insulin levels by 50%-60% on the fasted day (Heilbronn, et al., 2005). However, Studies involving overweight and obese non-diabetic humans have shown greater Improvements in insulin sensitivity in fasted individuals compared to their non-fasted, calories -matched counterparts. (Harvie, et al.,2011; Harvie, et al., 2013). After 5 weeks of observations, it was proven that early time restricted feeding improved beta-cell responsiveness. (Sutton, et al., 2018). On the other side, Intranasal oxytocin reduced fasting insulin secretion without affecting glucose levels, which also implies insulin -sensitizing properties of oxytocin. (Lawson, et al., 2015; Thienel, et al., 2016). Obesity is also

associated with oxytocin receptor knockout in mice, but it remains to be determined why this effect is observed only in male, but not female animals. (Takayanagi, et al., 2008).

2.5 Fasting and oxytocin effect on weight loss:

Fasting resulted as expected in marked weight loss with reduction of abdominal circumference, which was more pronounced in the groups who fasted longer. (Françoise, et al., 2019). On the other side, Pilot data suggest that longer -term oxytocin administration may support weight loss in obese patients. (Zhang,etal., 2013;Thienel,etal.,2016).

2.6 Fasting and oxytocin role in the treatment of cancers,

Fasting has been shown to improve the therapeutic responses of a variety of rodent cancer models, including gliomas, to chemotherapy (Antunes, et al., 2018; safdie, et al., 2012).2-3 consecutive days of fasting were effective against a wide range of cancers, particularly in combination with chemotherapy (Lee, et al.,2012; Bianchi, et al., 2015).Alternate day fasting performed in middle aged mice for a total of 4 months , also found that fasting reduced the incidence of lymphoma, bringing it from 33%(for control mice) to 0% (in fasted animals) (Descamps, et al., 2005). Interestingly, fasting on its own suppresses tumor growth in mice, but the greatest therapeutic response is found when fasting is combined with chemotherapy (Lee, et al., 2012). A reduction in food intake decreased the incidence of cancer in rodents (Rous, et al., 1914). Although fasting can reduce side effects associated with chemotherapy without negatively impacting tumor cell killing, fasting is not feasible for all patients, especially the elderly or those exhibiting cachexia. (Levine, et al., 2014; Tinkum,et al., 2015).However, Interestingly, emerging evidence has linked oxytocin to somewhat conflicting roles in carcinogenesis, as oxytocin is implicated in either fostering development or, conversely, inhibition of cancer related cellular functional phenomena.(Lerman, et al., 2018).Oxytocin has also been postulated to have a role in inhibiting proliferation of human cancer cells, which may offer protective role in preventing cancer initiation. (Cassoni, et al., 1994). The inhibitory role of oxytocin has been tested in individual site-specific cancers, such as human breast cancer and ovarian cancer (animal model). (Cassoni, et al., 1994; Murrell, et al., 1995; Morita, et al., 2004). A recent study reported that oxytocin, selectively activated by peptidylglycine, alpha -amidating monooxygenase (PAM), may play a role in preventing and controlling a small cell lung cancer. (Cao, et al., 2011). Oxytocin inhibited the proliferation, migration and invasion of ovarian cancer by enhancing the expression of E-cadherin and

slightly inhibiting MMP-2 in cultured skov3 cells. (Morita, et al., 2004). Oxytocin induces apoptosis in cultured three ovarian cancer cell lines including SKOV3, MDAH-2774 and PEO1. (Mankarious, et al., 2016) Oxytocin suppresses the ovarian cancer metastasis by repressing not only the expression of MMP-2, but also angiogenesis in cultured A2780 cells. (Hauyi, et al., 2018).It will be important to determine whether periodic fasting or its combination with a variety of therapies can be effective in re-sensitizing a wide range of resistant cancer cells. (Novella, et al., 2018). However, Oxytocin also shows promise to be used as cancer adjutant therapy, but it needs to be studied further (Ali, et al., 2018).

CHAPTER THREE: METHODOLOGY

3.1 Study design –

In order to determine the change that occur in oxytocin levels during fasting, Lnpep a gene that is involved in its metabolism in the liver was considered. Its levels of expression were to be monitored under different conditions. Data was obtained from the NCBI Gene Expression Omnibus (GEO) database, two datasets were selected for this study, (accession numbers GSE137385 and GSE130127). From the different GSEs, extrapolation of the following dataset information was done: run, assay type, library layout, organism, platform, strain, tissue and treatment as show in table 1. From GSE130127, selected were 9 runs beginning SRR89371 which is represented as X followed by the last 3 digits from each run as shown from (table 1) through the anlysis of the results. In the first group AL had 3 samples (X153, X154,X155) , in the second group IF16h3wks 3 samples were selected (X158,X159,X160), in the third group IF24h3wks 3 samples were selected (X163,X164,X165) and only 3 samples were selected from GSE137385, the 3 runs beginning with SRR10112 which also was represented as X followed by the last 3 digits from each run as shown in (Table 1), thus the fourth group 21fast 3 samples were selected (X565,X566,X567). A total of 12 samples were selected for comparing the different time points i.e (fasting for 21 hour a one day fast 21fast, fasting for 16 hours for 3 weeks IF16h3wks, fasting for 24 hours for 3 weeks) IF24h3wks and the the fed condition 0 hours of fasting AL.

The authors of GSE137385 declared that the dataset constitutes; Male mice at 8 weeks of age that were purchased from the Jackson Laboratory and trained for 1 week for a well-timed fasting and feeding protocol. On the last day of training, after fasting for 21 h, mice were sacrificed at 0, 3, 6 and 12 h following feeding and their livers were isolated and snap frozen in liquid N_2. Total RNA was extracted. RNAseq ~ 30 million reads, 150 bp pair-ended were obtained. (Chi Y, et al.,2020: Ng GY et al., 2019)

The authors of GSE130127 declared that the dataset constitutes; Mice that were randomly assigned, and subjected to ad libitum (AL), IF for 16-hour (IF16) and 24-hour (hereby termed as every-other-day (EOD)) for a period of three months. After which, genome-wide transcriptome analysis was performed using RNA sequencing in the liver. (Chi Y, et al.,2021).

3.2 Data acquisition.

Using Sequence Read Archive Toolkit the different runs show in table 1 were downloaded, and fastq files were obtained for each sample. (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software and the SRA Toolkit Development Team.)

3.3 Transcriptome data mapping and differential expression analysis

Quality Assessment (QA) of raw reads using FastQC and MultiQC, (Philip, et al.,2016) was performed to check the overall sequence quality, the GC percentage distribution (the proportion of guanine and cytosine bp across the reads) and the presence or absence of overrepresented sequences. Reads were then aligned using Hisat2(http://daehwankimlab.github.io/hisat2/manual), indexing using mouse genome sequence and annotation Release M10 (GRCm38.p4) (https://www.gencodegenes.org/mouse/release_M10.html). Read count summarization was done using HTSeq 2.0 (Putri et al.,2022). Differential Expression Analysis was done in R version 4.2.1 using DESeq2. The method used assume the data are on a log2 scale. Principal Component plot was obtained as shown in **figure 1**, Enhanced volcano plot on the differentially expressed genes was obtained as shown in figure 3. (Love et al.,2014).

3.4 Heatmap generation and enrichment analyses

To create heatmaps of differentially expressed genes, R and the R package pheatmap were used along with the log2Fold-Change output from R- studio R version 4.2.1 and results represented in figure 2 . To assess the biological significance of gene expression changes, GO enrichment analyses were conducted. GO enrichment analysis, focused on biological processes of differentially expressed genes was implemented by the GOfuncRs R package. GOfuncRs was installed in R and was used to test the statistical enrichment. Filtering of genes with adjusted P-value less than or equal to 0.03 were

considered significantly enriched by differentially expressed and from the results an extract of the Lnpep gene was obtained as shown in table2.

CHAPTER FOUR: RESULTS.

4.1 Deferentially expressed genes and clustering;

At first, a Principal component analysis (PCA) to study the gene differentiation patterns under the different conditions was performed that showed that biological replicates have high similarity to each other within AL, IF16h3wks and IF24h3wks groups through occupation of unique cluster regions , while for the 21fast a scatter plot that showed significant divergence from samples of the same treatment group was noted as an outlier (Figure 1). However, unique cluster regions representing each condition showed minimal overlap, suggesting that transcriptome patterns of AL, IF16h3wks and IF24h3wks largely differ from that of 21fast (Xiaoying et al.,2020).

Unsupervised hierarchical clustering of global RNA transcripts sequenced revealed distinct segregation of AL, IF16h3wks , IF24h3wks and 21fast groups under 4 separate clusters (Figure 2). This demonstrated that both IF24h3wks and 21fast induces differential expression of transcripts as compared to AL and IF16h3wks . Moreover, the AL transcriptomic pattern appears to be more similar to IF16h3wks than to IF24h3wks and 21fast. Despite this observation, F24h3wks and 21fast groups were discriminated after clustering, which reinforced the finding that the patterns of gene expression were still largely distinct between IF24h3wks and 21fast consistent with the PCA analysis (Figure 1).(Chi Y, et al.,2020).

Volcano diagrams showing the distribution of deferentially expressed genes in IF16h3wks , IF24h3wks and 21fast groups in comparison with the AL group. Transcriptome analysis of mice liver tissue revealed significant differences in gene expression in all groups compared to AL which was used as the control. This revealed that Lnpep gene was significantly up regulated as shown in (Figure 3).

Differentially expressed genes for all the conditions were analyzed with GO enrichment focused on related biological processes in order to elucidate the expected biological changes of the Lnpep gene. Selected terms of interest show that Lnpep gene was up-regulated (table 2) in which the Lnpen gene

plays a role in Molecular function relating to Protein binding and biological processes relating to Signal transduction.

4.2 Expression of the Lnpep gene with in the fasting and non fasting groups

Here, a plot of normalized counts against all groups AL, I 21fast ,F16h3wks and IF24h3wks groups was obtained, From the Plot of normalized counts for "ENSMUSG00000023845"(figure 4), the number of normalized counts for Lnpep gene represented as (ENSMUSG00000023845) which is its gene_id are detected to have elevated to highest abundance in the 21fast and the least detection was founded in the AL. The lowest number of normalized counts detection in IF16h3wks has been observed to be approximately equal to the maximum detection in AL. Detection in IF24h3wks remind significantly higher than those in AL. However, in all the three different fasting conditions, a detection of about 40 normalized counts elevation was observed which is also significantly higher than the maximum detection in AL.

Table 1: Showing the two datasets form GEO which were selected for this study, (accession numbers GSE137385 and GSE130127), sample run which were selected for the study, assay type, library layout, organism, platform, strain, tissue and treatment all included below.

accession numbers	**Run**	**Assay Type**	**Library Layout**	**Organism**	**Platform**	**STRAIN**	**Tissue**	**Treatment**
GSE130127	SRR8937153	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	ad libitum (AL) for three months
GSE130127	SRR8937154	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	ad libitum (AL) for three months

GSE130127	SRR8937155	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	ad libitum (AL) for three months
GSE130127	SRR8937158	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	intermittent t fasting (IF) for 16-hr (IF16) for three months. (IF16h3wks)
GSE130127	SRR8937159	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	intermittent t fasting (IF) for 16-hr for three months. (IF16h3wks)
GSE130127	SRR8937160	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	intermittent t fasting (IF) for 16-hr for three months (IF16h3wks)
GSE130127	SRR8937163	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	intermittent t fasting

								(IF) for 24-h (termed as every-other-day (EOD)) for three months. (IF24h3wks)
GSE130127	SRR8937164	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	intermittent fasting (IF) for 24-h (termed as every-other-day (EOD)) for three months. (IF24h3wks)
GSE130127	SRR8937165	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	intermittent fasting (IF) for 24-h (termed as every-other-day (EOD)) for three months. (IF24h3wk

								s)
GSE137385	SRR10112565	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	BALB/c	liver	Fasted for 21h. (21fast)
GSE137385	SRR10112566	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	Fasted for 21h. (21fast)
GSE137385	SRR10112567	RNA-Seq	PAIRED	Mus musculus	ILLUMINA	C57BL/6	liver	Fasted for 21h. (21fast)

Table 2: Showing Selected GO enrichment analysis results of the Lnpep gene obtained using GOfuncRs in R were filtering of genes with adjusted P-value less than or equal to 0.03 were considered significantly enriched by differentially expressed.

Node Id	**Ontology**	**Node name**	**Gene**
GO:0005515	Molecular function	Protein binding	Lnpep
GO:0007165	Biological process	Signal transduction	Lnpep

Figure 1: Showing Principal component (PCA) analysis which discriminates the variance in a data set in terms of principal components. The two most significant principal components (PC2 and PC1) are displayed on the x- and y-axes, respectively. Principal component analysis discriminated AL, IF16h3wks and IF24h3wks into three unique cluster regions with 21fast showing a significant divergence from samples of the same treatment group.

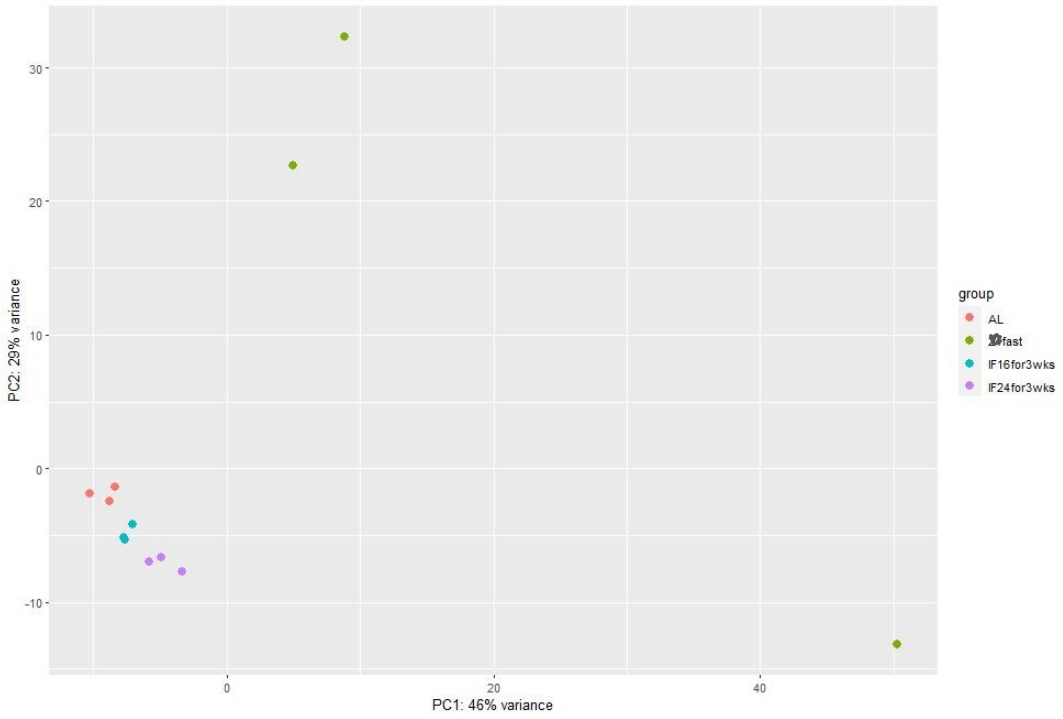


Figure 2. Showing cluster analysis of differentially expressed genes in four groups. Hierarchical clustering analysis was carried of all four groups under different experimental conditions, as indicated. mice under ad libitum feeding (AL), mice that fasted for 21 hours(21fast), mice under intermitted fasting for 16 hours in 3 weeks (IF16h3wks) and mice under intermitted fasting for 24 hours in 3 weeks (IF24h3wks). Unsupervised hierarchical clustering segregated **AL**, **IF16h3wks , IF24h3wks and 21fast** into four distinct cluster regions shown using a heatmap.**IF16h3wks** transcriptomic pattern appears to be more similar to **AL** than to **F24h3wks and 21fast**. Notably, one **21fast** sample appeared to be clustered segregated from its group replicates. Brown indicates high expression of genes, red moderate expression of genes whereas pink indicates low expression of genes.

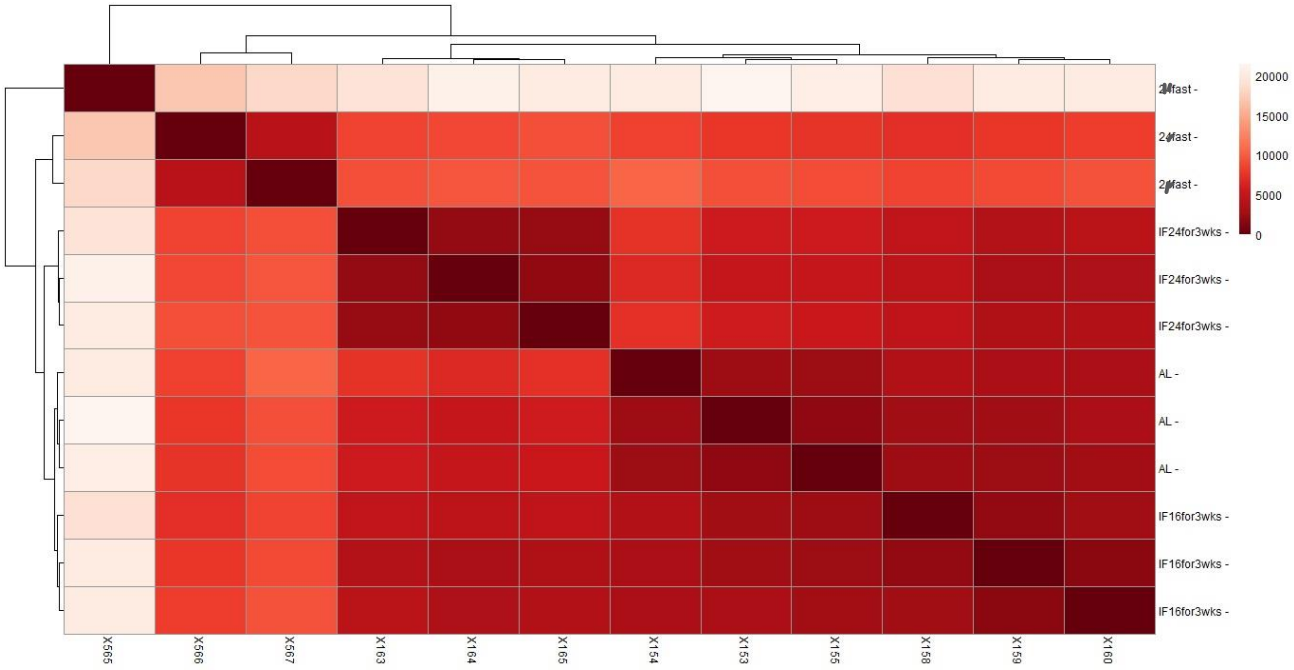


Figure 3: Showing EnhancedVolcano plot. Volcano diagrams showing the distribution of deferentially expressed genes in **IF16h3wks , IF24h3wks and 21fast** groups in comparison with the AL group. The threshold of differential expression is adjusted P-value ≤ 0.3 and fold change cut off = 0.05. The horizontal axis is the log 2 fold change of genes. The vertical axis is statistical significance scaled as #log 10 adjusted P-value. Each dot represents an individual gene (blue: no significant difference; red-right of the plot: up-regulated gene; red-left of the plot: down-regulated gene).marked is the position of Lnpep among the deferentially expressed genes which lies among the significantly up-regulated genes red- right of the plot.

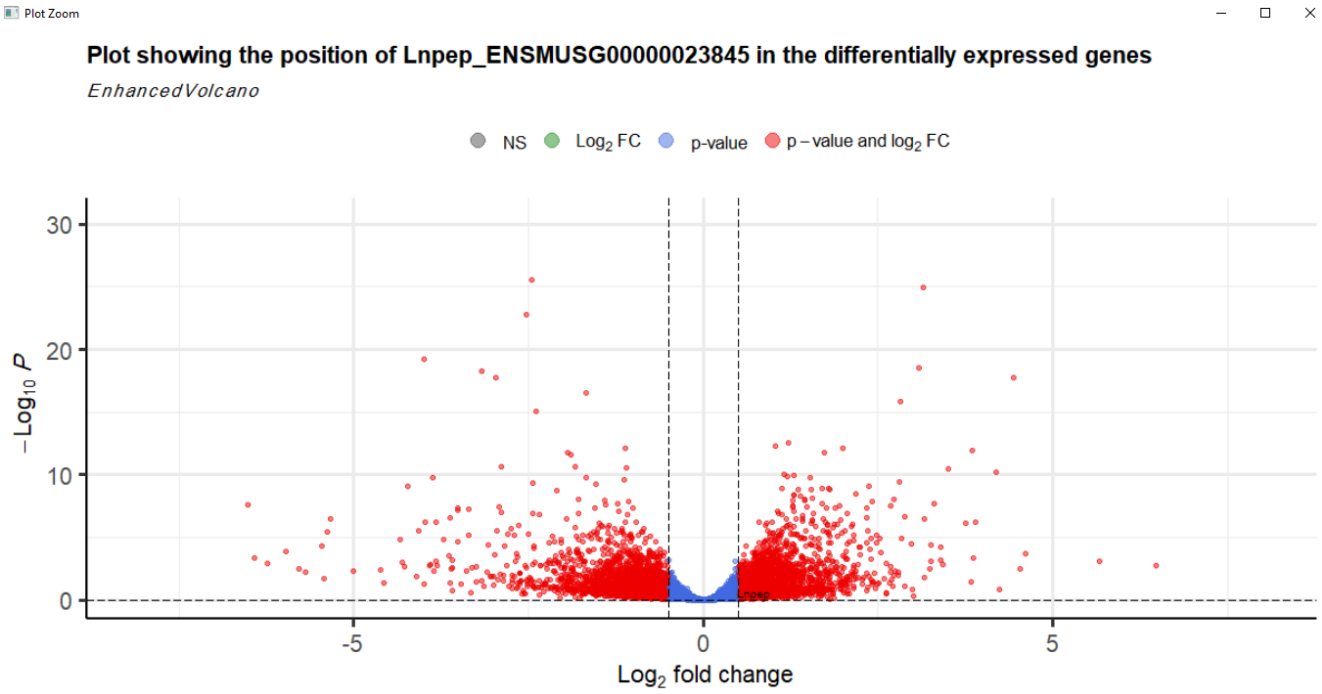
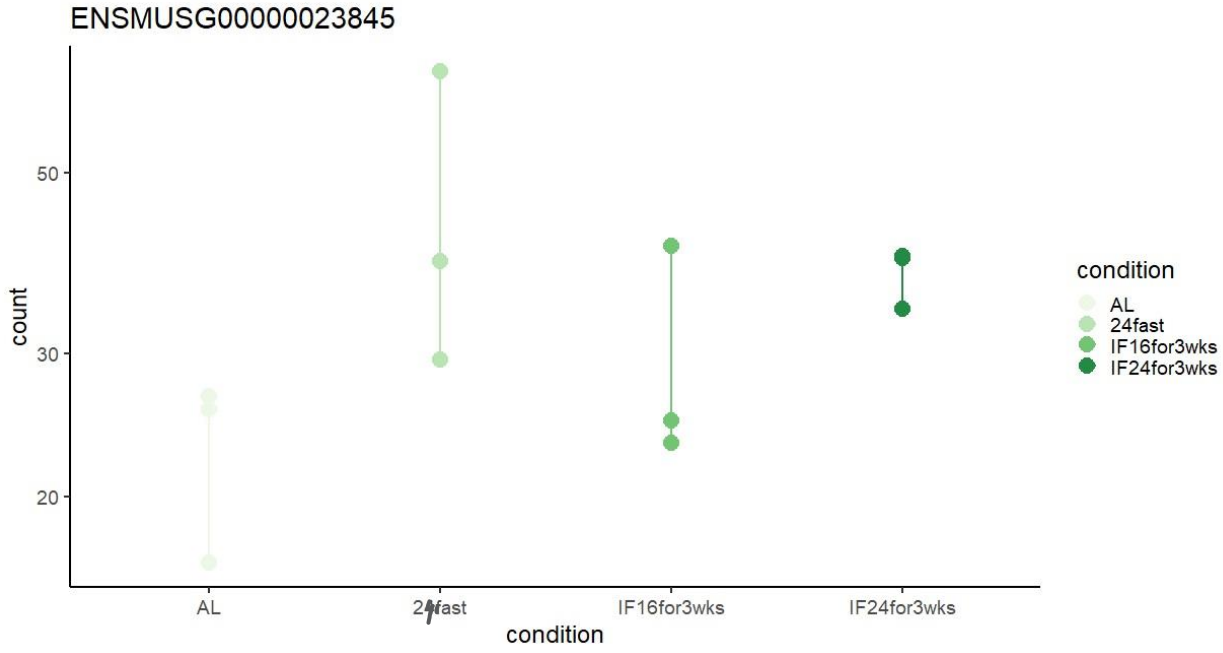


Figure 4: Plot of normalized counts for ENSMUSG00000023845 (Lnpep) for all the for groups showing the behavior of the Lnpep under the different feeding conditions that's mice under ad libitum feeding (AL), mice that fasted for 21 hours(21fast), mice under intermittent fasting for 16 hours in 3 weeks (IF16h3wks) and mice under intermittent fasting for 24 hours in 3 weeks (IF24h3



CHAPTER FIVE: DISCUSSION

The gene signatures exhibited by mice under ad libitum feeding (AL), mice that fasted for 21 hours(21fast), mice under intermitted fasting for 16 hours in 3 weeks (IF16h3wks) and mice under intermitted fasting for 24 hours in 3 weeks (IF24h3wks) indicate differentiation in gene expression according to the PCA, the abnormality seen in the 21fast may be due to the presence of the different mouse strains in the this group this resulted in the genes with expression levels significantly higher in C57BL/6J mice than in BALB/cJ mice and distinct clustering of experimental groups using heat map analysis. Even though common genes that are differentially expressed by in all fasting groups and AL are present, it can be further observed that a large number of differentially expressed genes are unique to either the 21fast or the IF24h3wks regimen. This shows that the longer the fasting time of the day the more genes are significantly expressed. (Gavin et al., 2019)

To examine the impact of fasting on of the Lnpep gene expression , an enhanced Volcano plot, was plotted $\log_{10}$P versus log2(-fold change) with an adjusted (pvalue ≤ 0.3) and fold change cut off = 0.05 considered significant, this was used to determine differentially expressed genes under the different conditions from mice that fasted for 21 hours(21fast), mice under intermitted fasting for 16 hours in 3 weeks (IF16h3wks) and mice under intermitted fasting for 24 hours in 3 weeks (IF24h3wks) was done in comparision with mice under ad libitum feeding (AL) as control. Lnpep gene was significantly upregulated with a (pvalue = 0.0853935 and adjusted pvalue = 0.2522642) this can be seen in the red zone right of the plot. This show that the Lnpep gene is levels increased significantly under different fasting conditions. Further study was done using the gene ontology (GO) enrichment analyses to determine its biological relevance, this revealed that Lnpep gene is involved in Molecular function and biological process, which is shown in table 2. The upregulation of Lnpep gene to significant levels implies an increase breakdown in oxytocin hormone with in the mice liver, thus proving true the hypothesis that oxytocin levels raise during fasting.

From the Plot of normalized counts for “ENSMUSG00000023845”(figure 4), the number of normalized counts for Lnpep gene represented as (ENSMUSG00000023845) which is its gene_id are detected to have elevated to highest abundance in the 21fast and the least detection was founded in the

AL. This means the highest increase in oxytocin levels was observed in the 21fast group while the minimum oxytocin levels were observed in the Al group. The lowest number of normalized counts detection in IF16h3wks has been observed to be approximately equal to the maximum detection in AL. For this case it shows that fasting for 16 hours may not bring about the desired oxytocin levels need to conduct the beneficial effects observed during fasting. Detection in IF24h3wks remind significantly higher than those in AL. This shows that fasting for 24 hours increases oxytocin levels to significant levels that may bring about the desired benefits of fasting. However, in all the three different fasting conditions, a detection of about 40 normalized counts elevation was observed which is also significantly higher than the maximum detection in AL. This shows that during fasting, there is increase in oxytocin levels to a threshold level which brings about the benefits observed during fasting Oxytocinase (Lnpep) plays a key role in the clearance of oxytocin during fasting (Elkins et al.,2017). These findings provide evidence of an association between fasting and oxytocin breakdown in the liver were elevated levels of the Lnpep gene implies high oxytocin clearance, thus high oxytocin levels. All fasting groups show similar elevation in oxytocin levels as noted of about 40 normalized counts. This could be the required threashold oxytocin level that brings about the beneficial effects observed during fasting.

Fasting resulted as expected in marked weight loss with reduction of abdominal circumference, which was more pronounced in the groups which was fasted longer (Françoise, et al., 2019). On the other side, Pilot data suggest that longer -term oxytocin administration may support weight loss in obese patients. (Zhang,et al., 2013;Thienel,etal.,2016). These finds provided evidence that fasting longer could be similar to longer-term oxytocin administration. Since a particular level of oxytocin is attained when an individual fasts thus, as one fasts longer this dose of oxytocin is attained, thus bring about a similar effect observed in fasting and oxytocin administration.

CHAPTER SIX: CONCLUSION.

These results provide evidence that there is a direct relationship between fasting oxytocin synthesis levels in mice. It was noted that there is threshold oxytocin level which was observed in all fasting groups and this could be the required oxytocin level need to bring about the beneficial effects observed during fasting. However, this calls for further studies to monitor oxytocin synthesis using brain tissue as to understand which exact genes involved in its synthesis are up regulated and to what extent during different fasting conditions.

RECOMANDATION

A 24 hour fast would be effective in attaining the desired oxytocin threshold levels however since it might not be easy to fast for such longer hours in humans, administration of oxytocin maybe used to reduce side effects associated with chemotherapy but also, Combination fasting and oxytocin administration could provide an effective therapy in re-sensitizing a wide range of resistant cancer cells, but this needs to be studied further.

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