EVALUATING THE LIPOGENIC EFFECTS OF SACCHARINE AND SUCRALOSE ON LIPID ACCUMULATION IN FATTY ACID EXPOSED HEPG2 CELLS

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Contents

Abstract

Currently, approximately a quarter of the global population suffers from non-alcoholic fatty liver disease (NAFLD), a statistic expected to surge by 50% by 2030, presenting a significant health concern alongside the obesity epidemic. In an effort to combat this escalating prevalence, low-calorie sweeteners are often substituted for sucrose. This study aims not only to replicate previous research suggesting that sucralose may trigger the expression of lipogenesis-related proteins, promoting hepatic steatosis and NAFLD in HepG2 cells but also to explore the effects of saccharin and sucralose on NAFLD-induced hepatic steatosis. Using HepG2 cells and varying doses of sucralose and saccharin, this study evaluates whether these sweeteners accelerate oleic acid (OA)-induced lipid accumulation by using oil red o staining and spectrophotometer measurements. While the findings align with some existing literature by showing no enhancement of OA-induced lipid accumulation by sweeteners, they diverge from contrary evidence suggesting a negative impact of sucralose on the acceleration of hepatic steatosis development. High variability in the experiment's dataset underscores the importance of conducting cell viability and proliferation assays, ensuring an optimal effect size, and adhering to standardized protocols for consistent and reproducible results. Factors such as oleic acid toxicity, the metabolomic profile of HepG2 cells, and cell growth status are carefully considered to explore potential sources of variability and reasons for conflicting findings compared to previous evidence. A deeper understanding of the multifaceted effects of sucralose and saccharin on human physiology is crucial for clarifying the potential adverse health consequences of regular intake, which could either mitigate or aggravate the development of hepatic steatosis and significantly influence the progression of NAFLD.

1. Introduction

Currently, about 25% of the world's population suffers from NAFLD, a figure projected to rise by 50% by 2030, posing a significant health threat alongside the obesity crisis (Eguchi et al., 2020; Fleischman et al., 2014). In the United States and other Western nations, it's estimated that 20% to 30% of adults experience excessive fat accumulation located in the liver (Vassilatou, 2014). The liver plays a central role in overseeing energy metabolism and the movement of lipids throughout the body (Rui, 2014). Overconsumption of dietary fats can result in the accumulation of fat in the liver, a condition known as fatty liver, which may develop into nonalcoholic fatty liver disease (De Cól et al., 2024). In NAFLD patients, liver cells gather fat in lipid droplets (LDs), representing an initial phase characterised by the relatively harmless buildup of LDs, termed steatosis. This stage is frequently linked to type 2 diabetes and is acknowledged as part of the metabolic syndrome (Cohen, Horton, & Hobbs, 2011). NAFLD include a range of liver diseases, from basic steatosis to steatosis accompanied by different levels of tissue inflammation and fibrosis (Graffmann et al., 2016). Hepatic steatosis may present as a harmless, non-inflammatory state posing challenges for clinical detection. In this case, fatty change can be reversed without negatively impacting the organ. However, it may also be linked to non-alcoholic steatohepatitis (NASH), which can advance to severe liver disease and ultimately hepatocarcinoma (Browning & Horton, 2004). Ectopic fat accumulation in the liver is primarily due to insulin resistance and chronic inflammation in adipose tissue due to obesity, key factors in NAFLD development and progression (Perry et al., 2015).

It is becoming more evident that food choices significantly impact the course and treatment of diseases like NAFLD as research explores deeper into the processes behind these health conditions. Providing consumers with low-energy, sweet-tasting elements replacing glucose and sucrose is an approach that has been attempted to reduce sugar intake mimicking the taste of sugar and lower calorie consumption without sacrificing sweetness. These elements are well recognised for their capacity to induce perception of sweetness by activating the pathways that lead to the perception of sweetness (Chattopadhyay et al., 2011).

Saccharin, the first artificial sweetener, was created in 1879 at Johns Hopkins University. Its sweetness level is 200–700 times greater than sucrose and it is found in many goods, including soft drinks, chewing gum, candies, salad dressings, and non-food items like mouthwash, toothpaste, and prescription drugs (Cohen, 1986). On the other hand, sucralose is the most popular artificial sweetener worldwide. It made up about 30% of the $2.29 billion global market for low-calorie sweeteners (LCS) in 2016. Offering a sweetness potency of around 600 times that of sucrose, sucralose closely emulates both the temporal and taste characteristics of sucrose (Laffitte et al., 2016). The FDA and the European Food Safety Authority have both authorised sucralose, with recommended daily intakes of 5 mg/kg/d and 15 mg/kg/d, respectively (Maragkoudakis, 2017). Supporting this, the WHO has also established that a daily allowance of 15 mg/kg of body weight is a suitable consumption for this sweetener (World Health Organization, 2023).

Interestingly, multiple studies have shown that low-calorie sweeteners raised the levels of inflammatory factors in the liver, triggered infiltration of lymphocytes into the liver, and boosted the expression of genes related to liver fat production in rats (Bian et al., 2017; Dhurandhar et al., 2018; Sánchez-Tapia et al., 2020). Specifically, sucralose modified glucose tolerance and bile acid metabolism by altering the gut microbiota composition. (Uebanso et al., 2017; Suez et al., 2014). Recent findings propose that sucralose can activate the ER stress pathway (Park et al., 2019). Likewise, despite saccharin's perceived innocuousness, WHO has raised concerns by issuing an alert suggesting a potential association between saccharin consumption and systemic inflammation as well as metabolic diseases (World Health Organization, 2023).

In humans, hepatic steatosis involves the storage of triglycerides and free fatty acids within liver cells, including the excess buildup of oleic acid (OA). Oleic acid, classified as monounsaturated omega-9 fatty acid, is generated as the outcome of de novo fatty acid synthesis (Okamoto, Tanaka, & Haga,

2002). Human hepatoblastoma cell line HepG2 cells treated with OA develop morphological resemblances to steatotic hepatocytes (Janorkar et al., 2009). To quantify the accumulation of OA, Oil Red O (ORO), a fat-soluble diazo dye, is commonly used as a biochemical technique for staining triglycerides on tissue sections. The staining intensity, when measured using a spectrophotometer, provides a quantitative indication of the amount of lipid present in the tissue sample (Cui, Chen, & Hu, 2010). A recent study implied that sucralose might induce lipogenesis-related protein expressions to increase hepatic steatosis, suggesting it may cause NAFLD in HepG2 (Wu et al., 2022). This study aims to replicate this paper to assess the reproducibility of scientific findings and the reliability of experimental outcomes in cell biology and metabolism. In addition, this study also aims to contribute to advancing the understanding of NAFLD pathogenesis and the search for viable treatments by creating a quantifiable in vitro model of hepatic steatosis. To investigate the effects of saccharine and sucralose-induced hepatic steatosis in NAFLD, HepG2 cells were treated with different doses of sucralose and saccharine to evaluate whether they affect accelerating oleic acid (OA)-induced lipid accumulation.

2. Materials and Methods

2.1. Cell culture

The experiment utilized the hepatocellular carcinoma cell line HepG2 obtained from University of Dundee. Initially, a flask containing HepG2 cells was prepared as the starting culture. The cells were routinely cultured in 4.5 g/L high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). They were kept in a sterile humidified incubator (Panasonic IncuSafe CO2 Incubator) at 37°C in a 5% CO2 atmosphere to prevent desiccation, with sterile water at the bottom of the incubator. For passaging, cells were routinely checked for confluency and viability using a trypan blue exclusion assay (Gibco). Once cells reached approximately 80% confluency, they were detached using trypsin-EDTA solution (0.25%; Sigma-Aldrich) and counted using a hemocytometer. Cells were then seeded at a density of 2.1x10^6 cells in a 75cm^2 flask (ThermoFisher Scientific) to maintain consistent cell density across experiments.

2.1.1. Sweetener Effects in 6-Well Plate Cultures

HepG2 cells were seeded at a density of 0.3 × 10^6 cells per well in 6-well plates (ThermoFisher Scientific), with each well measuring 9.6 cm in diameter. Prior to treatment, cells were cultured in low-glucose Dulbecco's Modified Eagle Medium supplemented with varying concentrations of sucralose (Sigma-Aldrich) (0.1, 1, and 10 mM) or saccharine (Sigma-Aldrich) (0.05, 0.1, 0.5, 1, and 10 mM) and incubated at 37°C in 5% CO2 for 24 hours. Each experiment was independently replicated three times, with each repetition conducted on different days for each sweetener, ensuring reproducibility and consistency of the results.

Fat induction was initiated 24 hours after cell passaging. Cells were treated with 1 mL of a 0.5 mM OA solution, prepared from a stock solution of 50 mM OA (British drug house) in ethanol. Control cells were cultured without treatment with the ethanol and oleic acid mixture used in the experimental group. The cells could incubate for 24 more hours under the same conditions. Confluency of cells was assessed under a microscope (leica DMi 1 inverted microscope), and the assessment of lipid accumulation treatment commenced only when cells reached 80% confluency, ensuring uniform cell growth and response to treatments.

2.1.2 Oleic Acid Dose Response in Cell Culture

An additional experiment was conducted once using 6-well plates to evaluate the dose response to oleic acid. Cells were cultured at a consistent density and pre-incubated in low glucose DMEM without any sweetener. Following a 24-hour incubation period at 37°C in 5% CO2, cells were exposed to their respective doses of OA. Each dose of oleic acid (0.05, 0.1, 0.5, 1, and 10 mM) was administered to three separate wells, ensuring triplicate measurements for each concentration. Subsequently, they were maintained in a humidified incubator for 24 hours before undergoing treatment, mirroring the protocol used in the previous experiment groups.

2.2 Assessment of Lipid Levels and Triglyceride Quantification

In evaluating lipid levels and triglyceride quantification, ORO staining was used to detect lipid accumulation in HepG2 cells. A working solution of ORO was prepared by combining 60 ml of ORO stock solution with 40 ml of distilled water, followed by filtration. Following this, the cells underwent a series of steps. They were rinsed twice with phosphate-buffered saline (PBS; Thermofisher), and fixed with 4% paraformaldehyde (37% paraformaldehyde, Fisher chemical) for 10 minutes. Subsequently, they were washed twice with water and then with 60% isopropanol (Fisher chemical). Each well received 1 mL of the ORO solution and was then left to incubate at room temperature for 15 minutes. Following the removal of the ORO solution, the cells were rinsed three times with double-distilled water (ddH2O) until the solution turned clear. Lipid accumulation in the cells was observed under a light microscope, with the presence of red oil droplets indicating OA-induced steatosis.

For quantification, ORO was then extracted from the dried cells by incubating with 1 mL of isopropanol for 3 minutes. Subsequently, 200 µL from each well was transferred into a 96-well plate (ThermoFisher Scientific) for accumulation assessment. The absorbance of ORO staining was measured once for each well in the 96-well plate using a spectrophotometer (BioTek ELx800)at 490 nm. In the oleic acid dose-response experiment, cell viability was assessed using a trypan blue exclusion assay. A trypan blue check was performed on one randomly selected cell from each concentration to evaluate cell viability. The protocol followed for viability assessment was similar to routine passaging, with adjustments made for the use of 6-well plates instead of T-75 flasks.

2.3 Statistics

Statistical analyses were performed using JAMOVI software. A paired t-test was employed to compare mean absorbance between cells with and without oleic acid, evaluating the differences in fat accumulation. On the other hand, one-way ANOVA assessed variations in sweetener concentrations (sucralose or saccharine) in samples with and without oleic acid, assessing the impact of dose on oleic acid accumulation when combined with sweeteners. The response variable represented the difference in oleic acid presence for each sucralose dose. A significance level of $p < 0.05$ was set for statistical significance. Prior to comparisons, Skewness and Kurtosis tests ensured normal distribution in all experimental and control groups. An F test for heterogeneity of variance preceded the ANOVA to ensure robust analysis.

3. Results

3.1. Control for confluency differences

To ensure the reliability and validity of the results, I rigorously managed variations in cell confluence across treatment groups. This involved initially seeding an equal number of HepG2 cells into each culture plate to standardize the initial confluency. Regular microscopic monitoring was conducted to

assess confluence levels, revealing consistent cell growth across all groups due to consistent seeding and treatment timing each week. Visual documentation confirmed no significant differences in confluence among treatment groups prior to ORO staining.

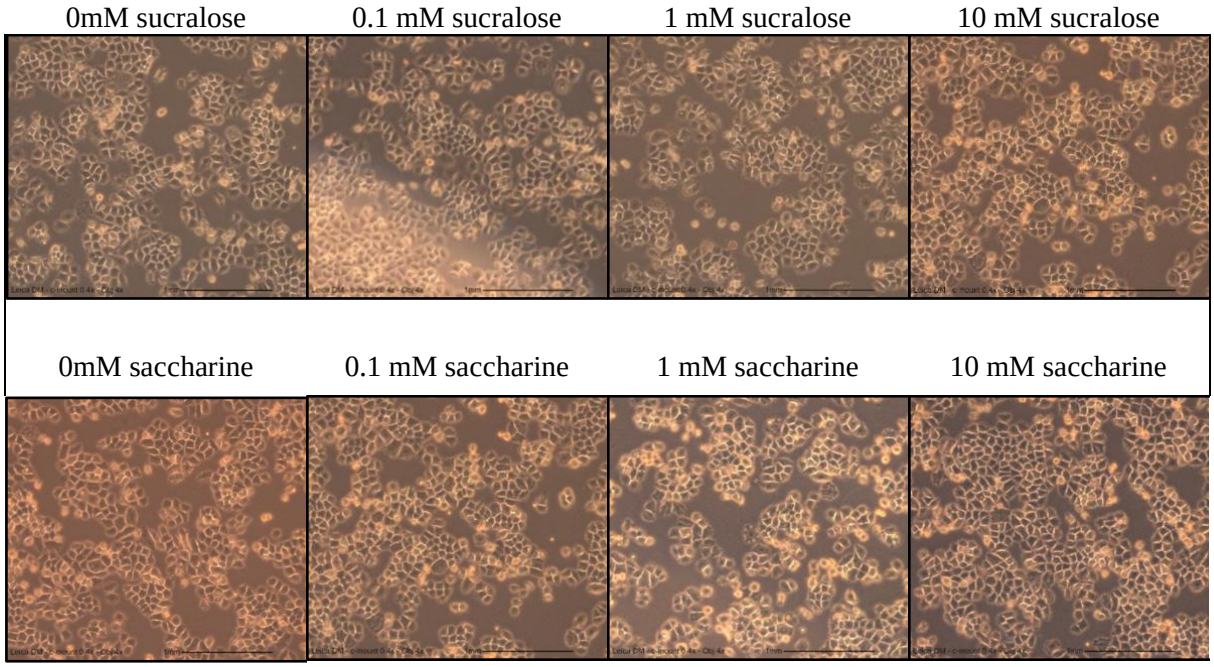
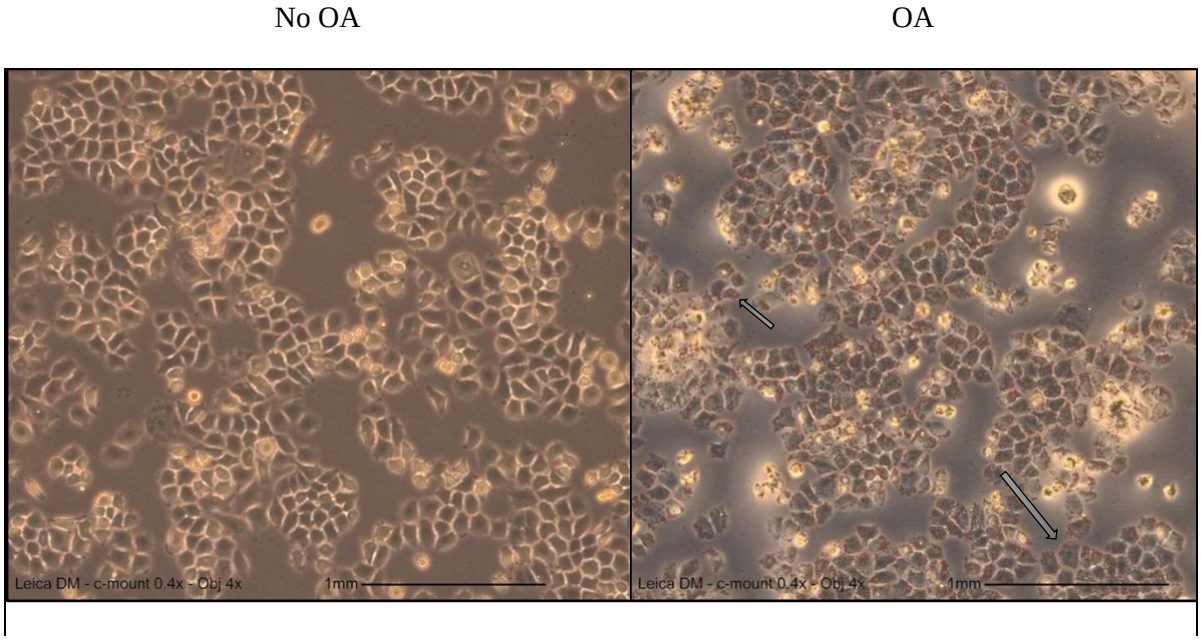


Figure 1: Representative images of HepG2 cell confluence rates across different treatment groups and concentrations, captured under a microscope prior to oil red O staining.

3.2 The Impact of 0.5 mM Oleic Acid Concentration on Absorbance

A paired t-test was employed to compare the difference in mean of cells treated with and without oleic acid.



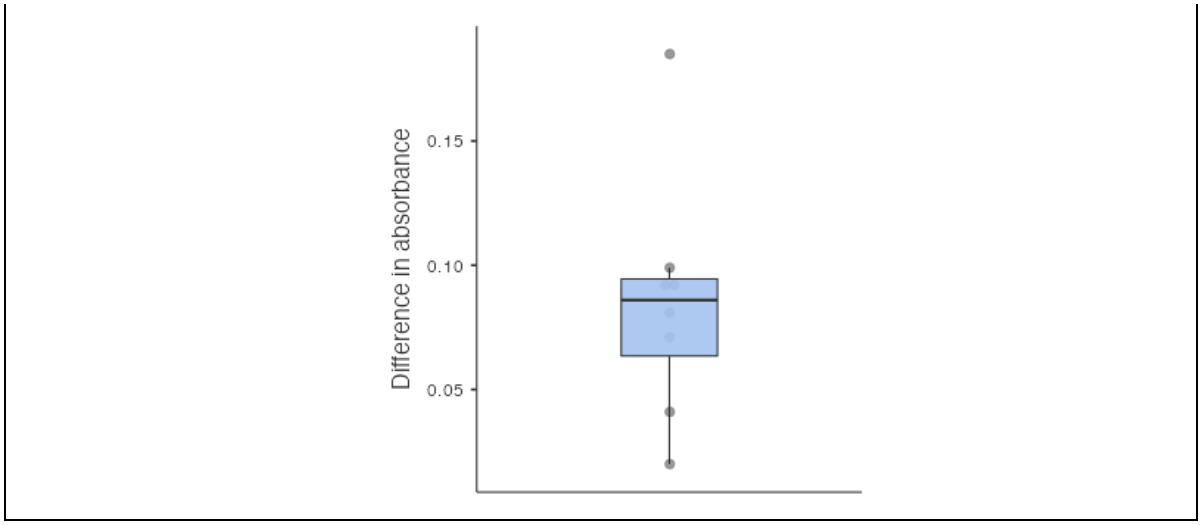


Figure 2: Lipid accumulation was assessed using ORO staining After 24 hours of seeding, HepG2 cells were exposed to 0.5 mM oleic acid (OA) for an additional 24 hours. Absorbance values were measured using a spectrophotometer. ORO selectively binds to fat deposits, resulting in red staining of lipid droplets, examples of these are indicated by the arrows. Box plot displays the distribution of data (n=6). Scale bars are 1mm.

Untreated HepG2 cells did not exhibit ORO staining. The pictures reveal that oleic acid enhanced lipogenesis to facilitate the accumulation of lipids in HepG2 cells. The addition of OA caused a significant 1.74 fold increase in absorbance compared to untreated cells ($T = 4.93$; $p = 0.0002$; $n = 6$). Therefore, a statistically significant increase in absorbance levels is seen when oleic acid was added compared to cells not treated with oleic acid.

3.3 The Dose Response to Oleic Acid

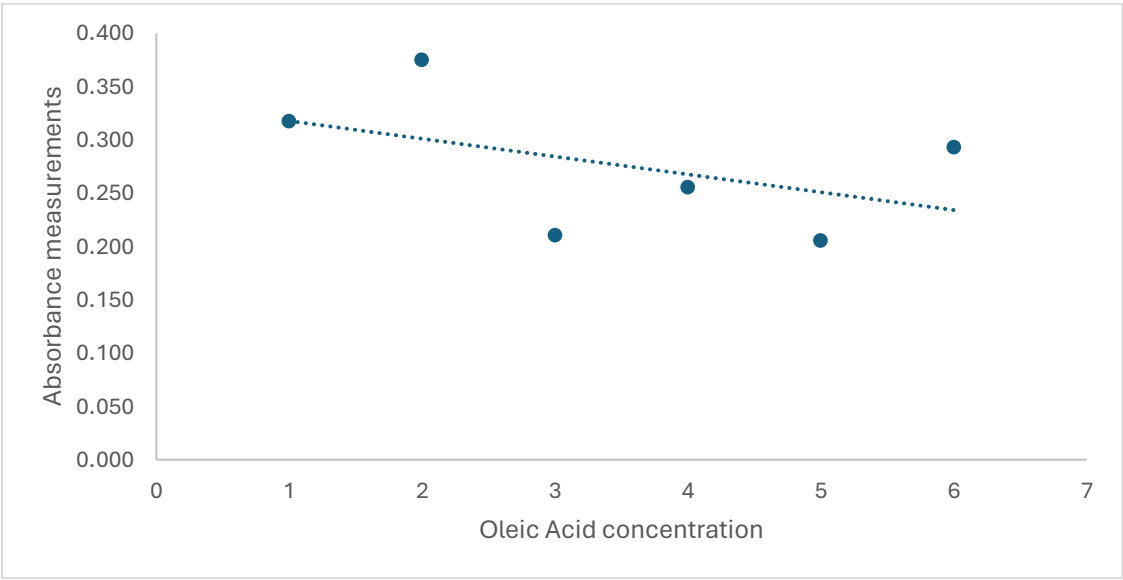


Figure 3: Absorbance levels following ORO staining of cells pretreated with various concentrations of OA (0mM OA, 0.5mM OA, 1mM OA, 2mM OA, 3mM OA, 5mM OA). Mean absorbance values were measured using a spectrophotometer. Data represents mean and linear regression (n =2).

Lipid accumulation did not show a linear pattern; higher concentrations of OA (0.5 to 3 mM) did not lead to increased ORO staining. The findings indicate that higher concentrations of oleic acid (OA) may not linearly affect absorbance levels post ORO staining. The highest absorbance was observed at a concentration of 0.5 mM OA. Consequently, this concentration was selected as produced maximal lipid accumulation.

3.4 Effect of Sweetener Dose on Absorbance Difference with and without Oleic Acid

3.4.1 Sucralose

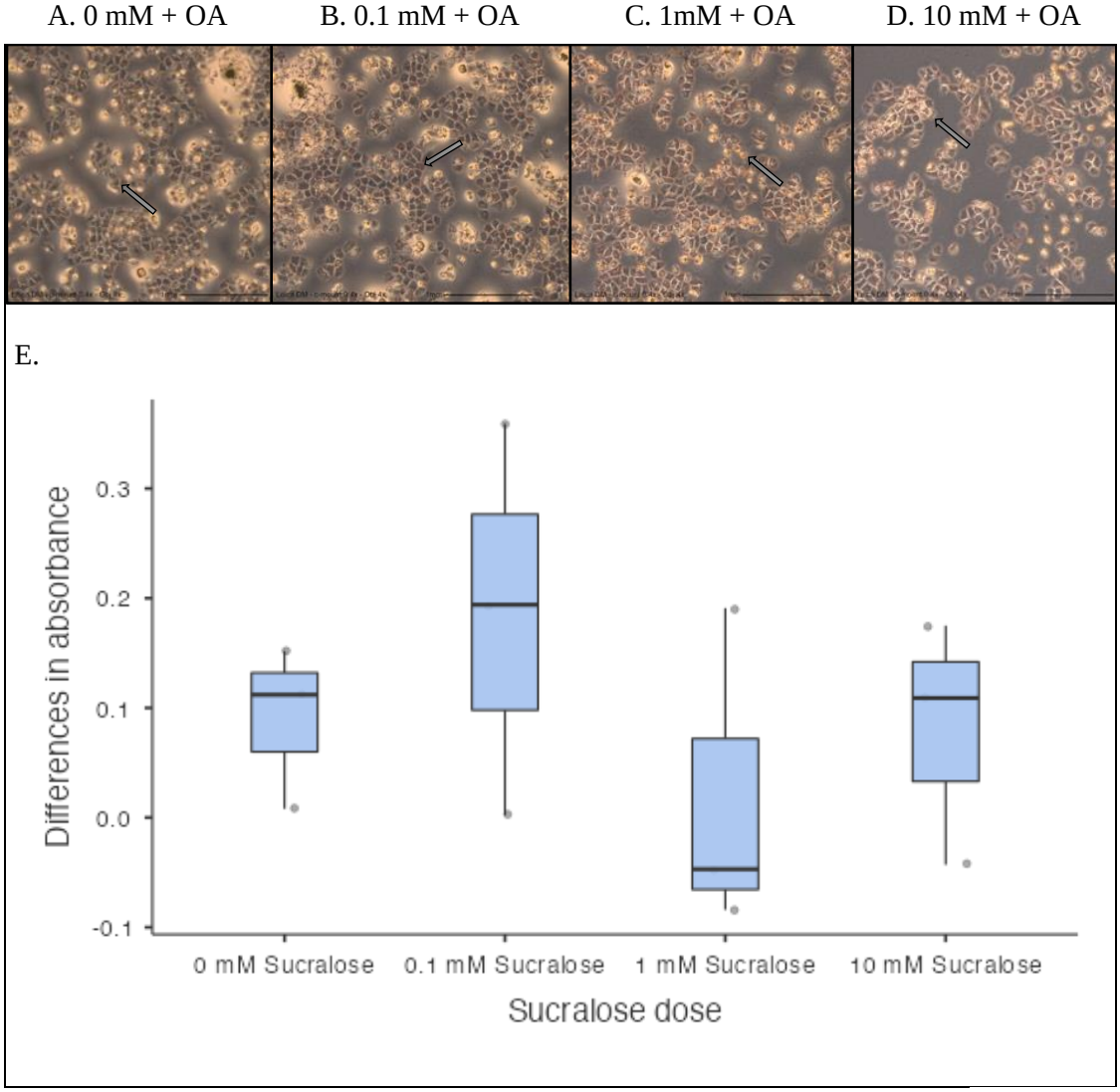


Figure 4: Lipid accumulation in HepG2 cells was assessed through ORO staining. ORO selectively binds to fat deposits, resulting in red staining of lipid droplets, examples of these are indicated by the arrows. Cells were pretreated with specified amounts of sucralose for 24 hours, followed by treatment with 0.5 mM oleic acid (OA) for an additional 24 hours. The resulting images (A-D) illustrate OA accumulation at each concentration. Absorbance values were measured using a spectrophotometer. The distribution of data is represented in the box plot (E) (n=3). Scale bars are 1mm.

Untreated HepG2 cells did not display ORO staining, indicating minimal lipid accumulation. Across sucralose concentrations, no significant difference in lipid accumulation was observed ($F(3, 4.22) =$

0.386, p = 0.769), with Levene's test confirming consistent variances (F(3, 8) = 0.722, p = 0.567). This suggests that sucralose did not promote lipogenesis or lipid accumulation in HepG2 cells.

3.4.2 Saccharine

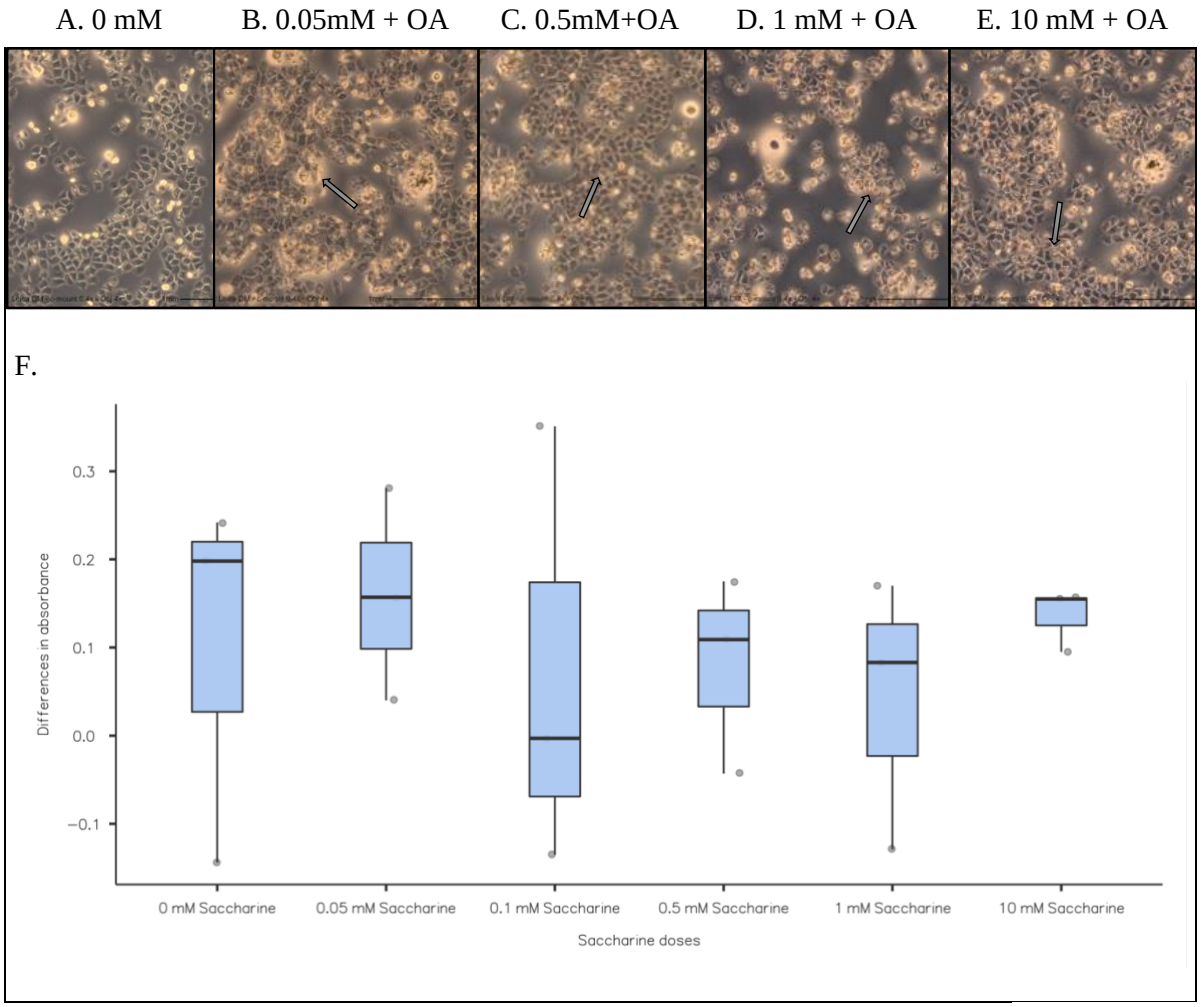


Figure 5: Lipid accumulation in HepG2 cells was assessed through ORO staining. ORO selectively binds to fat deposits, resulting in red staining of lipid droplets, examples of these are indicated by the arrows. Cells were pretreated with specified amounts of saccharine for 24 hours, followed by treatment with 0.5 mM oleic acid (OA) for an additional 24 hours. (A-E) The resulting images illustrate OA accumulation at each concentration. Absorbance values were measured using a spectrophotometer. (F) The distribution of data is represented in the box plot (n=3). Scale bars are 1mm.

Untreated HepG2 cells did not display ORO staining. Across saccharine concentrations, no significant difference in lipid accumulation was observed (F(5, 5.05) = 0.269, p = 0.912), with Levene's test confirming consistent variances (F(5, 12) = 2.21, p = 0.121). This suggests that saccharine did not promote lipogenesis or lipid accumulation in HepG2 cells.

4. Discussion

This study aimed to understand potential mechanisms triggered by sucralose and saccharine that might cause hepatic steatosis, the hallmark of NAFLD. HepG2 cells were tested with different amounts of sucralose and saccharine to evaluate their effects on oleic acid (OA)-induced lipid accumulation. This evaluation showed that both saccharine and sucralose expressed a lack of dose-dependent enhancement in OA-induced lipid accumulation. As expected, the addition of oleic acid significantly increased absorbance levels compared to untreated cells.

Prior research suggests that sucralose consumption has metabolic advantages, including weight loss and reduced hunger feelings (Higgins & Mattes, 2019; Peters et al., 2014). sucralose activates sweet taste receptors like T1R2 and T1R3 in taste and intestinal cells, crucial for detecting sweet substances within the gastrointestinal tract (Daly et al., 2021). It also boosts glucagon-like peptide-1 (GLP-1) release, aiding in blood sugar regulation, as seen in healthy individuals (Lertrit et al., 2018; Ahmad et al., 2020; Temizkan et al., 2015). Moreover, sucralose increases the expression of sweet taste receptors in the intestines and glucose transporters, leading to lower blood glucose levels. Compared to mice given sucrose, those receiving sucralose within acceptable daily intake levels showed reduced lipid accumulation, better plasma LDL-cholesterol levels, less hepatic fat deposition, and improved glucose tolerance (Wu et al., 2023).

Despite these findings, several studies have also highlighted negative metabolic impacts associated with sucralose consumption (Aguayo-Guerrero et al., 2024). In the study I aimed to replicate, sucralose was found to increase lipid buildup and significantly raise levels of lipogenesis-related proteins in animal and cell models (Wu et al., 2022). Similarly, long-term sucralose intake was linked to increased levels of blood LDL cholesterol, hepatic total fatty acids, and genes related to lipogenesis, consistent with earlier research (Qian et al., 2021; Suez et al., 2022; Wu et al., 2023; Sylvetsky et al., 2017). Therefore, the metabolic effects of sucralose are controversial, with the underlying mechanisms still not fully understood. However, several factors could contribute to this discrepancy.

The substantial variation in the data sets impacts the experimental outcomes. The variability in the effects of sucralose and saccharine on oleic acid-induced lipid accumulation across different days is unexpected, given the typically predictable growth rates of cells under similar conditions (Adan et al., 2016). This fluctuation in oleic acid accumulation over different days raises concerns about the reproducibility of the results. Despite efforts to maintain consistency using FBS, antibiotics, and uniform cell seeding, unexpected variability persisted, other underlying factors influenced outcomes. While protocol flaws are unlikely, given that any errors would manifest consistently across all cells, attention should focus on factors like oleic acid toxicity, HepG cell metabolomic profile, and cell growth status.

Kiseleva (2022) analyzed the effect of prolonged HepG2 cell cultivation on metabolomic profiles using GC × GC-MS. Variability in metabolites was observed over 20 days, observing findings both stable metabolic patterns and fluctuating features, emphasizing the dynamic nature of the metabolome (Kiseleva et al., 2022). Treating HepG2 cells, a human liver cancer cell line, with oleic acid (OA) produces liver cell-like features similar to fatty liver cells (Janorkar et al., 2009; Okamoto et al., 2002). Given the scarcity of human liver samples, HepG2 cells serve as a reliable substitute, mimicking hepatocellular steatosis while distinguishing metabolic effects from cell damage (Kanuri & Bergheim, 2013; Ricchi et al., 2009; Graffmann et al., 2016). Despite factors like culture conditions and experimental techniques influencing HepG2 behavior and experimental outcomes' variability, maintaining consistent culture conditions and conducting methodical assays can minimize these discrepancies (Kanuri & Bergheim, 2013). Consequently, HepG2 cells have become a common in vitro model for studying NAFLD (Müller & Sturla, 2019; Janderová et al., 2003)

Ensuring sufficient cell confluency before oleic acid treatment is crucial for promoting cell growth and metabolic activity. Variations in cell metabolism over time can significantly influence cell growth

dynamics (Adan et al., 2016). During the assessment of various oleic acid concentrations, a cell count was conducted, revealing a scarcity of cells. This count, performed just before the ORO staining treatment in the 6-well plates, adhered to the standard protocol for regular cell counts in T-75 flasks. Several factors could contribute to the low cell count. Ineffective trypsinization leading to incomplete cell detachment, excessive cell adhesion, or the presence of cell debris may explain the scarcity. Alternatively, the slow growth rate of the cells could also be a contributing factor (Fischer et al., 2021).

Despite their appearance of being densely packed under the microscope, the cells may not be proliferating as expected. Although there is no obvious difference in confluence among the treatment groups and concentrations by the time the ORO staining, further investigation is needed to identify the exact cause of the low cell numbers. Commonly assessed using an MTT assay, conducting a cell proliferation assay could provide insights into how sucralose and saccharine affect cell growth (Cui et al., 2005; Hu et al., 2003). The impact of steatosis on hepatocyte proliferation remains largely unexplored. Research using the MTT assay found that oleic acid (OA)-induced steatosis in HepG2 cells inhibited cell proliferation (Cui, Chen, & Hu, 2010). This was supported by microarray analysis (De Gottardi et al., 2007). While apoptosis induced by OA reached saturation at 1 mM, decreased proliferation was linked to increased p27 expression. Since p27 acts as a cyclin-dependent kinase (CDK) inhibitor, it hinders the cell cycle at the G1-S checkpoint, suggesting that OA-induced steatosis suppresses cell proliferation by inhibiting p27 expression, thereby impeding G1-S progression (Feldstein et al., 2003).

OA interacts with cell membranes, impacting their fluidity and permeability by inserting itself between phospholipid molecules. This alters the membrane's composition and organization, affecting its overall structure. These changes in membrane fluidity can also influence the function of membrane-bound proteins and enzymes, potentially disrupting their activities (Walker & Hadgraft, 1991). As a result, OA has been implicated in modulating cellular responses to oxidative stress and inflammation, which can further influence cell viability and apoptosis (Eynaudi et al., 2021). In studying OA-induced apoptosis mechanisms, various apoptosis-related proteins should be evaluated. Specifically, in HepG2 cells treated with OA, elevated levels of cleaved caspase-9 suggest activation of apoptosis pathways, while no noticeable changes in Bax or Bcl-2 expression are observed post-OA induction (Cui, Chen, & Hu, 2010). However, it's crucial to recognize that OA effects can vary depending on factors such as concentration, cellular context, and exposure duration. Notably, oleic acid demonstrates non-cytotoxicity at doses of 0.25mM and 0.5mM (Dibwe et al., 2024), consistent with findings in this study where lower doses correlate with higher absorbance and cell count.

Conducting viability counts aids in assessing cell mortality or membrane integrity disruption. A low cell count, or membrane disruption could indicate cell death as a potential contributing factor (Adan et al., 2016). The absence of a viability assay in the original paper is a notable gap identified during the replication attempt. Assessing cell survival through viability assays like MTT, Neutral Red assay, or trypan blue exclusion assay can offer valuable insights into different experimental factors that may affect cell viability (Riss et al., 2013). An experiment induced hepatic cellular steatosis in HepG2 cells using varied concentrations (0.5, 1.0, 1.5, and 2 mM) of OA solutions. Lipid levels were measured via absorbance at 517 nm post-ORO staining. Viability decreased significantly with OA concentrations >1.0 mM compared to controls (Yang et al., 2018). Despite the potential adverse effects of oleic acid, particularly at higher concentrations, this hypothesis is challenged by both the absence of similar observations at the initial stage of the study and the low cytotoxicity of OA at the employed dose (0.5 mM). Additional analysis of OA doses could optimize conditions for consistent lipid accumulation, with the inclusion of a cell viability assay to monitor cell health and ensure result consistency benefiting the experiment.

On the other hand, the staining technique may also contribute to the observed variability.ORO staining is a technique used to visualize fat in cells and tissues. It relies on the preferential solubility of ORO in neutral lipids compared to the staining solution (Ramírez-Zacarías et al., 1992; Levene et al., 2012; Catta-Preta et al., 2011). This method has been employed for over four decades to differentiate preadipocytes from adipocytes, primarily for qualitative analysis purposes (Green & Kehinde, 1974;

Kuri-Harcuch & Green, 1978). However, this staining method can be operator-dependent and consequently, interpretations may vary significantly (Cui, Chen, & Hu, 2010). Additionally, discrepancies exist among published protocols, particularly in concentrations, volumes, and durations, with limited performance data available (refer to the appendix for details on the protocol followed in this study). This inconsistency raises concerns about the reliability of the method. Despite referencing a common original protocol from 1992, variations persist among different quantitative ORO staining procedures (Ramírez-Zacarías et al., 1992; Kraus et al., 2016). The lack of information about the preparation of ORO reagents in the original paper posed a challenge for replication. Following the same protocol precisely is essential for consistency and reproducibility of results. Transparency in methodology enables a comprehensive understanding of the study's procedures and helps identify potential sources of variability (Kraus et al., 2016).

Oil red O staining has expanded to quantitatively analyze adipocyte differentiation (Kim et al., 2016) Elution of the dye from cells with 2-propanol and photometric absorbance measurement at 518 nm facilitate quantification (Ramírez-Zacarías et al., 1992). To quantify OA-induced steatosis in HepG2 cells objectively, fluorescence imaging, flow cytometry, enzymatic colorimetric assays, or GC-MS are recommended (Du et al., 2023; Lee et al., 2004). This study used absorbance measurement, offering a direct correlation with lipid concentration that facilitates quantitative analysis but it is also susceptible to variability in sample preparation and instrument settings, affecting the reproducibility and comparability of results between experiments and laboratories (Kanamori et al., 2021). As an alternative, a colorimetric assay utilizing ORO was used to quantify steatosis, based on its high organic solubility. After confirming the feasibility and optimizing conditions, the assay demonstrated high sensitivity and reproducibility, correlating well with OA-induced steatosis in HepG2 cells ($r2 = 0.97$, $p = 0.001$). The method accurately quantifies steatosis induced by OA doses ranging from 0.1 to 2 mM. This ORO-based assay offers a convenient, quantifiable, and reliable means to study NAFLD pathogenesis and therapy (Cui, Chen, & Hu, 2010).

Replicating a study requires careful attention to the effect size, as even minor differences in experimental conditions or cell characteristics can impact the observed effects. To effectively detect real differences in outcomes, it's crucial to consider the variability present and determine an appropriate sample size (Sullivan & Feinn, 2012). The lack of information on sample size and replication frequency in the original study suggests that our study may have had a smaller effect size, requiring a larger sample size for reliable results. This highlights the importance of considering effect size in both study design and interpretation. Increasing the number of replicates can enhance the statistical power and consequently, the reliability of results. However, determining the optimal sample size involves considering practical constraints and measurement sensitivity (Maher et al., 2013). While a formal power analysis was not conducted, observations suggest the potential need for larger sample sizes, particularly when variability is high. This realization underscores the importance of rigorous quantification of results, as qualitative assessments may overlook subtle variations like effect size and variability. Additionally, exploring dose-response relationships for sucralose and saccharin could provide valuable insights for future research (Cui, Chen, & Hu, 2010).

While glucose can stimulate TAS1R2/TAS1R3 at extremely high levels (>300 mM), most LCSs are notably stronger agonists, requiring lower concentrations (<100 μM) to activate these taste receptors (Zhang et al., 2010). Sucralose solutions in the micromolar range (with an EC50 of 39 μM) are enough to trigger TAS1R2/TAS1R3 activation (Servant et al., 2010). The original study by Wu et al., 2022 proposed that sucralose boosted hepatic lipogenesis via T1R3 in HepG2 cells. It's plausible that the HepG2 cells utilized in this study may have differed in taste receptor expression compared to those in the original study. To investigate this hypothesis, analyzing T1R3 expression levels through methods such as qRT-PCR or Western blotting would be necessary (Liu et al., 2020). Moreover, employing receptor knockdown methods to modify T1R3 receptor expression in HepG2 cells, such as siRNA-mediated knockdown or plasmid-based overexpression, could provide further insight (Kojima et al., 2015).

In humans, sweet taste perception involves T1R3 and T1R2 receptors, which together detect sweetness (Mahalapbutr et al., 2019). LCSs such as saccharin, acesulfame-K, and sucralose activate TAS1R3/TAS1R2 receptors, possibly influencing insulin release (Kyriazis et al., 2014; Roberts et al., 2000). Yet, the impact of LCSs on typical consumers remains unclear, mainly observed in vitro (Roberts et al., 2000).The activation of the sweet taste receptors triggers a series of signaling events, including the dissociation of gustducin and subsequent activation of phospholipase C β2, leading to a reduction in cyclic AMP activity (Thompson et al., 2014). This cascade triggers the release of calcium ions and initiation of TRPM5 channels, leading to transmitting taste signals to the brain and cell depolarization (Liu & Liman, 2003). Despite this understanding, there is a gap in knowledge regarding the specific pathways of different sweeteners on lipogenesis, as each type of low-calorie sweetener varies significantly in terms of pharmacological efficacy, pharmacokinetic profile, and chemical structure (Lee & Owyang, 2017).

Despite the gaps in understanding specific sweeteners and the concerning evidence of their potential to increase lipid accumulation in OA-induced scenarios, it is crucial to conduct further studies comparing the effects of LCSs and sucrose. Promising evidence suggests that using sucralose as a sugar alternative in daily consumption could benefit glycemic control (Wu et al., 2023). Ultimately, sweeteners act as substitutes for sucrose and may be a promising solution to help mitigating the high prevalence of metabolic diseases (Chattopadhyay et al., 2011).

Considering all factors, the inability to replicate the original study underscores the critical importance of transparency in methodology and results, as well as the need to carefully assess effect size. Moreover, incorporating cell viability and proliferation assays is essential to ensure reproducibility and enhance our understanding through replication. While the data indicate that sucralose and saccharine did not affect OA-induced lipid accumulation, evidence suggests potential negative effects on increased lipogenesis due to sweeteners. This contradictory result highlights the need for additional research to clarify the underlying mechanisms contributing to result variability. This will ultimately delineate the potential adverse health effects of regular sucralose and saccharine intake, either mitigating or aggravating the development of hepatic steatosis, and thereby profoundly impacting the progression of NAFLD.

5. Acknowledgements

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Appendix

Oil Red O Staining protocol

Cell preparation

- Culture and treat cultured cells in tissue culture plate as required.

Medium removal and cell washing

1. Take the 6 well plate out of the incubator and remove the media.

2. Add 2 ml of PBS to wash the cells, ensuring complete coverage, and remove PBS completely.

3. Wash the cells two times with 2 ml of PBS.

Fixation with 4% paraformaldehyde and isopropanol

4. Then add 2 ml of 4% formaldehyde (at room temperature) to the cells and incubate for 10 min at at room temperature. Do not add formaldehyde directly to the cells. Pipette onto the wall and mix gently rotating.

5. Carefully remove paraformaldehyde with a pipette. Dispose of the formaldehyde waste into a designated waste bottle labelled for formaldehyde disposal. When the waste bottle is full, contact the technicians for hazardous waste management.

6. Wash cells with 2 ml of distilled water twice. Discard waste into a designated waste container for biological waste.

7. Wash cells with 2 ml of 60% isopropanol for 5 min at room temperature.

8. Discard 60% isopropanol. Dispose of the isopropanol waste into a designated waste solvent bottle labelled provided by the technicians. When the waste bottle is full, contact the technicians for collection.

Staining with Oil Red O

9. Cover the cells evenly with 1mL Oil Red O Working Solution.

10. Rotate the plate or dish, and incubate for 10–20 minutes at room temperature.

11. Discard the Oil Red O solution. Dispose of the solution waste into a designated waste solvent bottle labelled provided by the technicians. When the waste bottle is full, contact the technicians for collection.

12. Wash the cells 3 times with water until no excess stain is seen.

13. Once the final wash is complete, carefully remove any remaining water from the wells using a pipette or by tilting the plate and dry excess liquid with a laboratory wipe.

14. Allow the plate to air dry open bench for approximately 5–10 minutes to ensure complete evaporation of residual paraformaldehyde and water before further processing or observation.

Microscopic observation

Cover the cells with water and view them under the microscope. Lipid droplets appear red and nuclei appear blue.

Quantification of Oil Red O Staining in liver cells

1. Take out the water, and after allowing the plate to air drying, add 1 ml of 100% isopropanol per well in the case of a 6-well plate.

2. Incubate at room temperature for 3 minutes to ensure proper extraction of the Oil Red O stain into the isopropanol solution.

3. Using a standard pipette, remove 200ul from each well and transfer it into a 96-well plate for quantification.

4. Measure the absorbance at 490 nm using a spectrophotometer.

5. Dispose of the isopropanol waste into a designated waste solvent bottle labelled provided by the technicians. When the waste bottle is full, contact the technicians for collection.

Reagents

1. Oil Red O Stock: FW 408.5. Weigh 0.35 g Oil Red O and put in 100 ml of isopropanol. Stir O/N, filter (0.2 μ) and store at RT.

2. Oil Red O Working Solution: Mix 60 ml of Oil Red O stock solution with 40 ml of ddH2O. Let sit at room temp for 20 min followed by filtering (0.2 μ).

3. 10% Formalin in PBS: Dilute 27 ml of 37% formalin stock solution in 73 ml of PBS.

4. 100% Isopropanol (Fisher chemical)

5. 60% Isopropanol: Mix 60 ml of 100% Isopropanol with 40 ml of ddH2O.