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THE EFFECTS OF PERSISTENT ORGANIC POLLUTANT DIOXIN ON REVERSE CHOLESTEROL TRANSPORT PATHWAY OF HDL IN DIFFERENTIATED 3T3-L1 ADIPOCYTES

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ABSTRACT

Cardiovascular disease (CVD) the leading cause of mortality globally occurs primarily due to coronary artery disease caused by hypertension, diabetes and dyslipidemia. In addition to the above-mentioned risk factors, persistent organic pollutants (POPs) which include dioxin also play a role in CVD. They are transported by lipoproteins and accumulate in adipose tissue with low-density lipoprotein (LDL) found to be the main transporter of lipid-soluble POPs. However, very-LDL (VLDL) and high-density lipoprotein (HDL) have also been associated with lipid-soluble POPs in humans. An in-vitro study looking at the effects of dioxin in reverse cholesterol transport (RCT) of high-density lipoprotein cholesterol (HDL-c), an anti-atherogenesis pathway was undertaken to further understand it's mechanism of action. **MATERIALS AND METHOD:** 3T3-L1 preadipocytes were differentiated into mature adipocytes according to manufacturer instructions. A cholesterol efflux capacity assay was performed using mature adipocytes loaded with fluorescence-labelled cholesterol and treated with dioxin concentrations of 1,10,30 nm and dioxin 30 nm together with rosiglitazone (0.005 μmol/ml). The movement of labelled cholesterol from the cells to the acceptor (HDL) was quantified with the intensity of fluorescence-labelled cholesterol measured at 485 nm excitation and 523 nm emission. **RESULTS AND DISCUSSION:** The percentage of cholesterol efflux using dioxin concentrations of 1 and 10 nm and HDL was lower than that of HDL alone. However, the highest Dioxin concentration (30 nm) showed comparable results with HDL after a 4 hours efflux incubation period. Dioxin together with rosiglitazone and HDL showed unexpected results with a lower percentage of cholesterol efflux. This shows that Dioxin and rosiglitazone did not significantly increase the ability of HDL to promote cholesterol efflux in mature adipocytes as it should function. **CONCLUSION:** These findings suggest that dose of Dioxin exposure, to some degree, influences cholesterol efflux which warrants further exploration.

Keywords: Persistent Organic Pollutant Dioxin; Reverse Cholesterol Transport; HDL, Cardiovascular disease; Cholesterol efflux

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality globally with 17.9 million people dying annually from CVD in 2019 (WHO). CVD occurs primarily due to atherosclerosis which is triggered by hypertension, diabetes and dyslipidemia (1,2). Recently, it has been shown that persistent organic pollutants (POPs), which include Dioxin, influence metabolic diseases that increase the risk of atherosclerosis. POPs are transported by lipoproteins and accumulated in adipose tissue with low-density lipoprotein (LDL) found to be the main transporter of lipid-soluble POPs (3). Human can be exposed to Dioxin through the consumption of contaminated food, water, air and skin absorption. Very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) have also been associated with lipid-soluble POPs in humans (4,5). However, the effect of Dioxin on cholesterol transporters is not established and hence investigated in this study.

Cholesterol efflux is the process by which excess cholesterol is removed from cells, primarily through a class of proteins called ATP-binding cassette transporters (ABCA1 and ABCG1) and high-

density lipoprotein (HDL) particles, often referred to as “good cholesterol”. This process is essential for preventing them accumulation of cholesterol in cells and by extension, the development of atherosclerosis (6,7,8).

Dioxin is a group of toxic chemical compounds that can have a wide range of adverse effects on human health. While much of the research on dioxin has focused on its carcinogenic properties and impact on the endocrine system, it can also affect various aspects of lipid metabolism, including cholesterol transporters. Cholesterol transporters are essential for regulating cholesterol levels in the body, and disruptions in their function can lead to various cardiovascular and metabolic problems (6). Dioxin primarily exerts its effects on cholesterol transporters through its influence on gene expression.

It is important to note that the effects of dioxin on cholesterol transporters are complex and can vary depending on factors such as the dose and duration of exposure, as well as individual genetic susceptibility. Additionally, the health consequences of dioxin exposure extend beyond cholesterol transporters to include a wide range of adverse effects on the cardiovascular system, immune system, and overall health. Reducing exposure to dioxin and other environmental toxins is an important step in minimizing these health risks.

MATERIALS AND METHODS

3T3-L1 preadipocytes were cultured into adipocytes according to manufacturer instructions. Fully differentiated 3T3-L1 adipocytes (Day 15) were then cultured with a treatment medium containing DMEM, 10% FBS, and 1% penicillin/streptomycin antibiotic to proceed with the next experiment. Cells were maintained at 37°C in a humidified 5% CO2 incubator.

Lipid content was measured using a commercially available kit (AdipoRed Assay Reagent). Test experiments were done on undifferentiated 3T3-L1 preadipocytes, differentiated 3T3-L1 adipocytes on day 9, and fully mature 3T3-L1 adipocytes on day 15 to see the progress of lipid accumulation in the cells. For qualification test, the adipocytes (Day 9) and the matured adipocytes (Day 15) was tested for the presence of lipid contents by staining the cells with AdipoRedTM assay reagents. For quantification assay, lipid content was measured by AdipoRedTM assay, cells were washed with PBS and 100 µL of PBS was added to the wells. AdipoRed reagent (30µL) was added to each well. After 10 min, the fluorimeter and fluorescence were measured with an excitation wavelength of 485 nm and emission wavelength of 572 nm.

For the MTS assay (Cell Proliferation, Colometric, Abcam, UK), on day-15, the adipocytes cell was cultured in the treatment medium containing various concentration of dioxin (0.1nM, 1nM, 10nM, 30nM and 60nM) and cells were incubated in 37°C incubator, 5% CO 2 for 24 hours, 48 hours and 72 hours. Cells with adipocytes medium were served as control meanwhile, cells treated with 0.5ul toluene in 100ul medium were served as vehicle control. After 24 hours, 48 hours and 72 hours incubation, 10ul MTS was added into each well and returned to the incubator for 3 hours. The plate was shaken and absorbance was measured at 490 nm using a microplate reader (Perkin Elmer Victor X5 2030) to determine the formazan concentration, which is proportional to the number of viable cells.

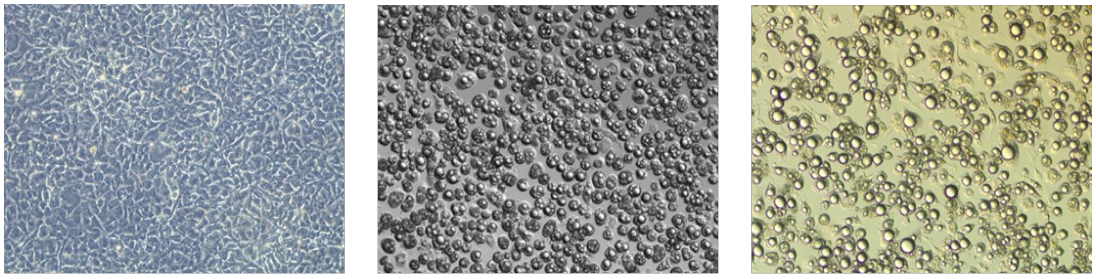
For the quantative measurement of cholesterol efflux assay (Cholesterol Efflux Assay Kit, Abcam, UK, Cell-based), after mature adipocytes ready, they were loaded with fluorescence-labelled cholesterol, labelling medium for 1-hour incubation, followed by an equilibrium process, in which cells were incubated in equilibration medium to equilibrate the labelled cholesterol among all intracellular cholesterol pools for 24 hours. After that, the cells were treated with dioxin (1, 10 & 30 nm) and dioxin (30 nm) together with rosiglitazone (0.005 µmol/ml) then incubated with cholesterol acceptors and the movement of labelled cholesterol from cells to the acceptor was quantified. The capability HDL (100 µg/ml) to take up labelled cholesterol was determined after 4h of efflux incubation period. TNF-Alpha (10 ng/ml) was used as a positive control while toluene as vehicle control. The intensity of fluorescence-labelled cholesterol was measured at 485 nm excitation and 523 nm emission.

All experiments were conducted at least in triplicate, and the data are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA, followed by Tukey post hoc test for multiple comparisons. A p-value of less than 0.05 was regarded as statistically significant for differences between groups.

RESULTS AND DISCUSSION

3T3-L1 Cell culture and treatment

3T3-L1 preadipocytes was cultured into full adipocytes according to the manufacturer instruction. Briefly, preadipocytes 3T3-L1 was cultured in 3T3-L1 Preadipocyte medium until confluent. Following this, cells were induced for differentiation by culturing in the 3T3-L1 differentiation medium for 3 days until fully differentiated. The fully differentiated 3T3-L1 adipocytes was cultured in the 3T3-L1 Adipocyte medium until matured (15 days). Based on Figure 1, the differentiation of 3T3-L1 from preadipocytes to mature adipocytes took 15 days after growth in the differentiation medium.



Preadipocytes cells | Day-9 Adipocytes cells | Day-15 Adipocytes cells

Figure 1: Cells images with 10x magnification. The differentiation of 3T3-L1 from preadipocytes to mature adipocytes took 15 days after growth in the differentiation medium.

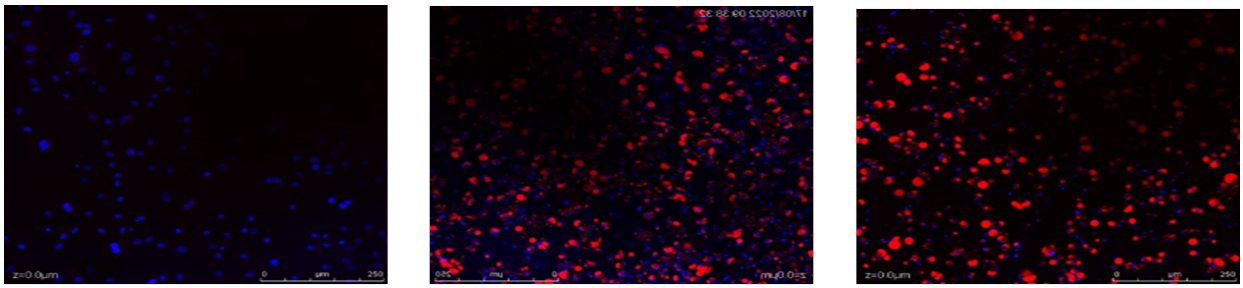
Mature adipocytes and pre-adipocytes are two different phases of fat cell development that differ significantly in structure, function, and appearance. In term of shape and size, pre-adipocytes are undifferentiated cells that have a fibroblast-like shape and relatively smaller and have a more elongated or spindle-shaped appearance.

On day 9, the cells are in an intermediate stage, transitioning from pre-adipocytes toward mature adipocytes. They are smaller in size, with an elongated or fibroblastic shape, and have a high nuclear-to-cytoplasmic ratio. There are few lipid droplets, as the cells have not yet accumulated significant fat. The cells are actively undergoing differentiation and preparing for the transition to mature adipocytes.

By day 15, mature adipocytes are larger and more spherical. Adipocyte development is characterized by lipid accumulation, and as the cell stores energy as fat, its shape becomes rounder compare to day 9. The cells have a distinct appearance with a centrally located, flattened nucleus pushed to the periphery by the large lipid vacuole.

Qualification of lipid content by AdipoRedTM assay

High secretion of lipid droplets is one of the potent characteristics of mature adipocytes. This can be observed within the incubation period of preadipocytes to mature adipocytes**.**



Preadipocytes cells | Day-9 Adipocytes cells | Day-15 Adipocytes cells

Figure 2: Image of Adipored (red) and nuclues staining (blue) indicating accumulation of lipids in Adipocyte cells. Images were taken by Confocal Laser Scanning Microscope and observed under 20x objective.

In pre-adipocytes, the nucleus is centrally located and occupies a significant portion of the cell. The cell is more metabolically active, with processes related to cell division, signaling, and differentiation. In mature adipocytes, the nucleus is flattened and displaced to the periphery of the cell due to the large lipid droplet. The nucleus in mature adipocytes is less metabolically active compared to pre-adipocytes.

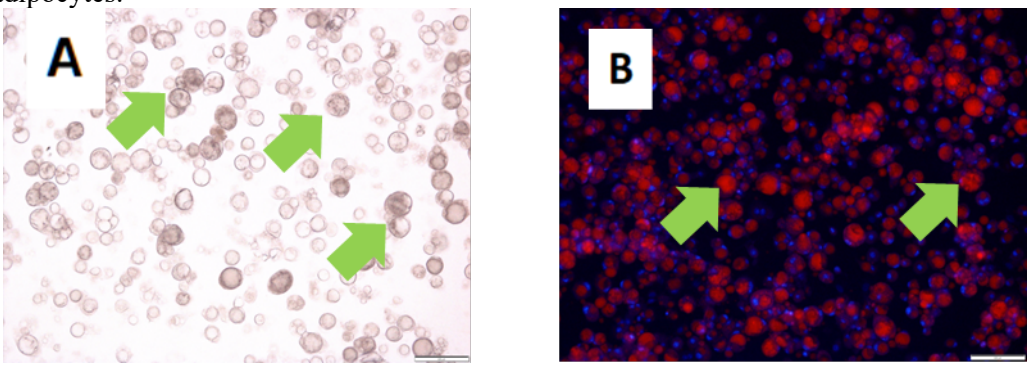


Figure 3: Cells images with 10x magnification. A: Bright field and B: Fluorescence. Cells were stained with Adipored reagent(red) and DAPI for nucleus (blue). Arrows are the lipid droplets.

This figure showed mature adipocytes day 15 with different technique of imaging. The rounder shapes are larger and more spherical, filled with large lipid droplets containing triglycerides. Intracellular triglyceride accumulation is frequently utilized as a measure of adipocyte development. The lipid droplets keep getting bigger and more numerous.

Quantification of lipid content by AdipoRedTM assay

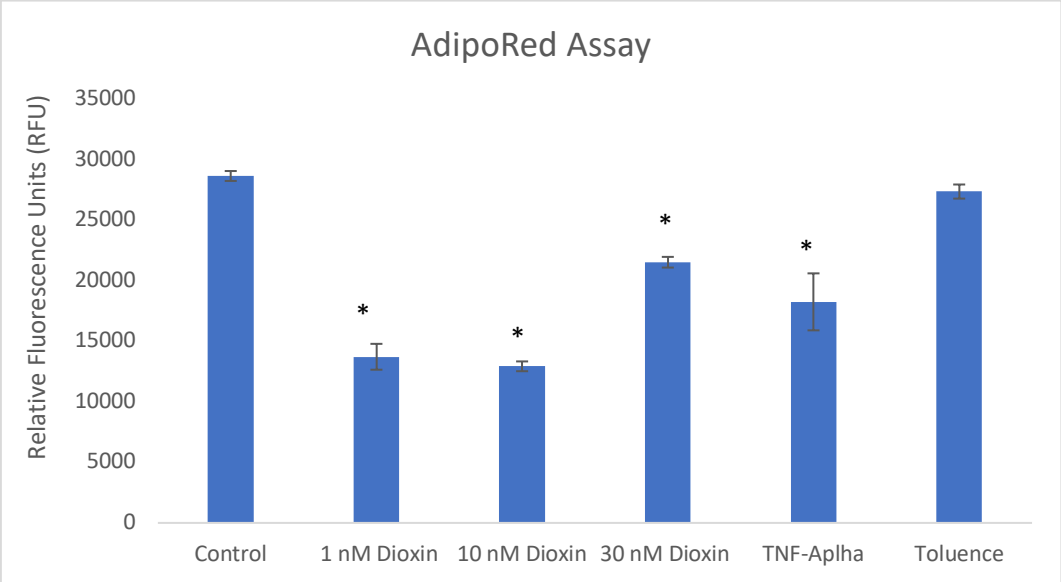


Figure 4: 3T3-L1 cells were induced to differentiate in the presence of dioxin with 1, 10 and 3 nm, TNFα, toluene and no inhibitor. Lipid accumulation was assayed after 15 days mature adipocytes. Data are expressed as Mean + SEM (n=5), *p<0.05 versus control group.

Based on the result above, there are no significant different between and within each group. The accumulation of lipid droplets of dioxin treated with 1,10 and 30 nm of Dioxin representing significant reduction compared to the control. Same goes to TNF-alpha which acts as positive control. This suggests that Dioxin at this concentration decreases or reducing lipid accumulation. The lipid content increases to 21,549 at 30 nM Dioxin, indicating a reversal of the dose-dependent effect at higher concentrations. This could be because of toxicity changing the metabolism of lipids or a compensatory cellular response. TNF-Alpha showed the lipid content with 18,273, lower than the control but higher than the effects seen with Dioxin (1 nM and 10 nM). TNF-alpha seems to suppress lipid accumulation, although not as strongly as Dioxin. While toluene showed the lipid content which is close to the control level. This suggests that Toluene has little to no effect on lipid accumulation.

MTS Cell Viability Assay

MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay) is a commonly used colorimetric assay for assessing cell viability, proliferation, and cytotoxicity in biological experiments. For this assay cell were exposure to dioxin which was detected using MTS Assay Kit with different concentration of dioxin and times. This assay aims to reveal the effect of the dioxin in different concentration and incubation time on cell proliferation and cytotoxicity, affecting the cell viability.

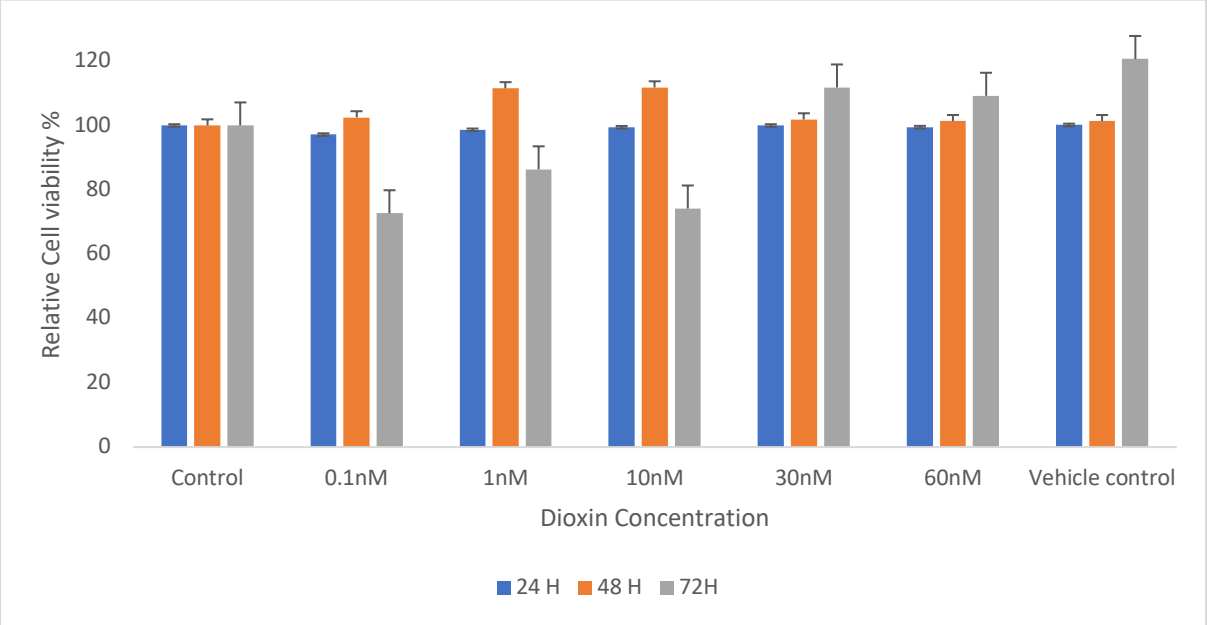


Figure 5: Cell viability assay of 3T3-L1 cells exposed to different concentrations of dioxin (0.1 nM to 150 nM) with different time incubation.

Based on the results above, viability assay of 3T3-L1 cells exposed to different concentrations of dioxin (0.1 nM to 150 nM) for 24 hours, 48 hours and 72 hours incubation showed no toxicity effect. However, dioxin with concentration of 0.1, 1 and 10Nm showed significantly decrease after 72h while for higher concentration 30 and 60 nm. showed % cell viability increased.

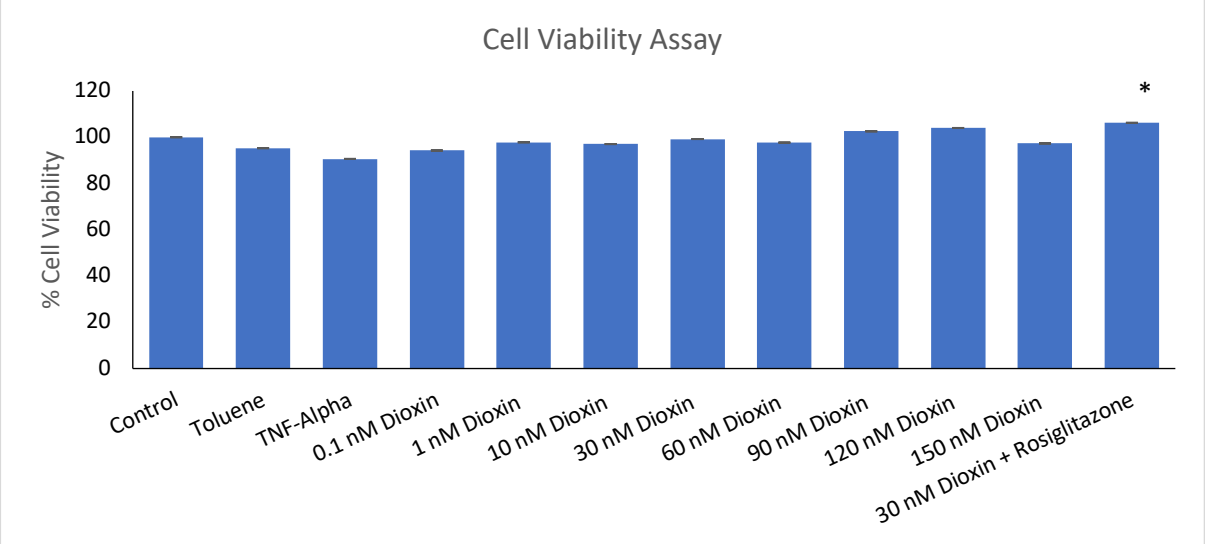


Figure 6: Cell viability assay of 3T3-L1 cells exposed to different concentrations of dioxin (0.1 nM to 150 nM). Graph represent average cell viability (mean SEM values, n=5, *p<0.05 versus control group).

From the result above, concentration of dioxin with 0.1 nM - 30 nM showed viability remains fairly stable with no strong indication of toxicity at these doses. While dioxin with 60 nM - 120 nM gave results with viability exceeds 100% which suggesting potential stimulatory effects at higher doses.

However, dioxin with concentration 150 nM showed viability decreased may due to that extremely high doses might lose the stimulatory effect or begin to introduce toxicity. The combination treatment with Rosiglitazone at 30 nM Dioxin shows the highest viability at 106.23%, suggesting a potential protective or enhancing effect from this drug.

Cholestrol Efflux Assay

The movement of labelled cholesterol from the cells to the acceptor (HDL) as control group treated with 1,10,30 nM of Dioxin and dioxin 1, 10 and 30 nM together with Rosiglitazone (0.005 µmol/ml) plus HDL for each treatment were quantified with Cholesterol Efflux Assay Kit (Cell-based, Abcam, UK) at 485 nm excitation and 523 nm emission.

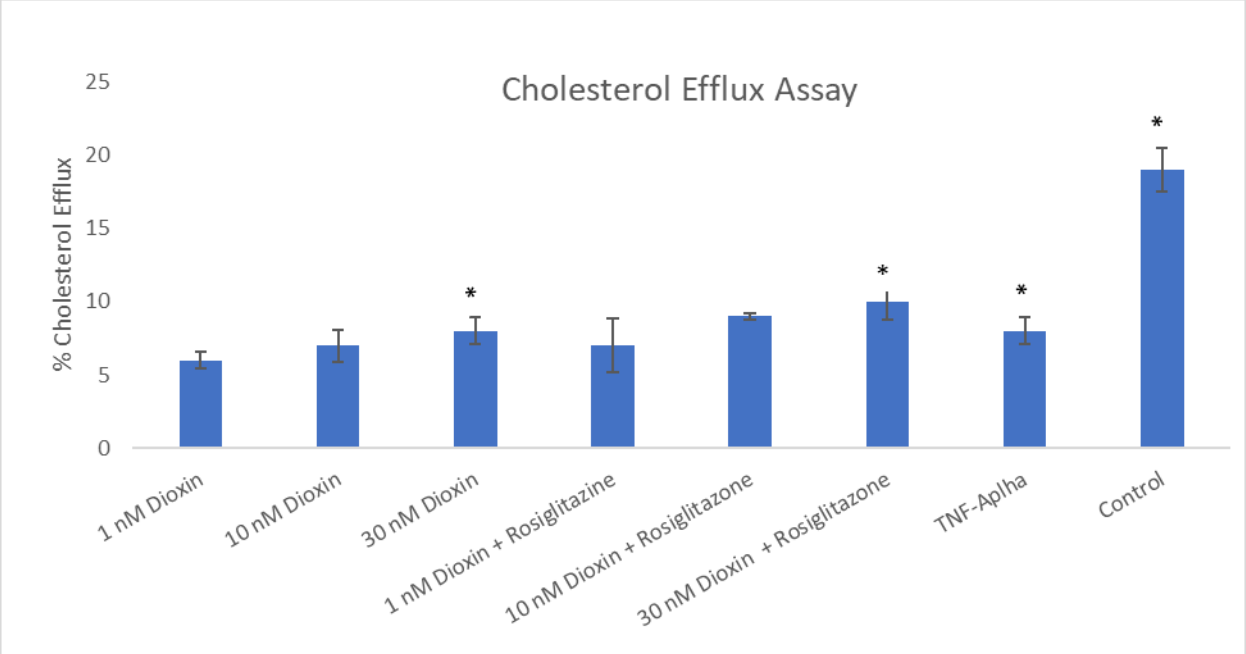


Figure 7: Cholesterol Efflux Assay was quantified in cells exposed to 1,10,30 nM of Dioxin and Dioxin 1, 10 and 30 nM together with Rosiglitazone (0.005 µmol/ml) plus HDL for each treatment. Graph represent % cholesterol efflux (mean SEM values), n=4, *p<0.05 versus control group.

3T3-L1 cells were treated with differentiation concentration of dioxin, dioxin together with rosiglitazone, HDL as acceptors (Control) for cholesterol while TNF-Aplha served as a positive control. The percentage of cholesterol efflux using dioxin concentrations of 1, 10 & 30 nM and increased within 4-hour efflux incubation time. Among the dioxin groups, concentrations 30 nm showed the highest percentage of cholesterol efflux followed by 10 nm and 1 nm. However, the highest Dioxin concentration (30 nM) showed with significantly different compared to control and TNF-Alpha after 4 hours efflux incubation period. Treatment dioxin together with rosiglitazone showed higher percentage of cholesterol efflux compared dioxin alone. Dioxin 30 nm together with rosiglitazone showed significantly different compared to control and TNF-Alpha after 4 hours efflux incubation period. All other groups did not show any significant differences.

Rosiglitazone acts as a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, which regulates genes involved in glucose and lipid metabolism. Its effect or may induce on cholesterol efflux. This is primarily mediated by the enhanced activity of ATP-binding cassette (ABC) transporters, such as ABCA1, which play a crucial role in the efflux of cholesterol to HDL particles. It is also suggested that rosiglitazone can alter the lipid composition of HDL particles. This change might improve the function of HDL in cholesterol efflux, even if the total HDL cholesterol levels are not significantly altered. Based on the trend, looks like incubation of 1, 10 and 30 nm dioxin with Rosiglitazone have a higher trend in increasing cholesterol efflux compared to Dioxin alone. However, comparisons involving "Dioxin plus Rosi" groups did not show significant differences. This suggests the effects of "Rosi" treatment are not dramatically different at these levels.

CONCLUSION

Dioxin at 1, 10 and 30 nm had lower cholesterol efflux compared HDL alone. Interestingly, incubation of dioxin with Rosiglitazone exhibits higher trend of cholesterol efflux capacity however not as potent as HDL alone. Therefore, it can be postulated that PPAR gamma pathway is responsible for the mechanism of dioxin inhibiting cholesterol efflux capacity in 3T3 L1 adipocytes.

REFERENCES

[1]. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)

[2]. Alice Dupont Juhl and Daniel Wüstner (2022). Pathways and Mechanisms of Cellular Cholesterol Efflux—Insight From Imaging. Front. Cell Dev. Biol., 01 March 2022
Sec. Membrane Traffic Volume 10 - 2022 | https://doi.org/10.3389/fcell.2022.834408

[3]. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015. I.World Health Organization. ISBN 978 92 4 156516 5

[4]. WHO (2002). Polychlorinated dibenzodioxins, polychlorinated dibenzofurans, and coplanar polychlorinated biphenyls. In: Safety evaluation of certain food additives and contaminants. Geneva, World Health Organization (WHO Food Additives Series, No. 48; http://www.inchem.org/documents/jecfa/jecmono/v48je20.htm).

[5]. Cave, E. & Crowther, N.J. (2019). The Use of 3T3-L1 Murine Preadipocytes as a Model of Adipogenesis. In: Guest, P. (eds) Pre-Clinical Models. Methods in Molecular Biology, vol 1916. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-8994-2_25

[6]. Jie Luo, Hongyuan Yang & Bao-Liang Song (2020). Mechanisms and regulation of cholesterol homeostasis. Nature Reviews Molecular Cell Biology volume 21, pages225–245

[7]. Krause BR & Hartman AD. (1984). Adipose tissue and cholesterol metabolism. Lipid Res; 25:97-110.

[8]. Zhang, Y., Mc Gillicuddy, F.C., Hinkle, C.C., O'Neill, S., Glick, J.M., Rothblat, G.H. & Reilly, M.P. (2010). Adipocyte modulation of high-density lipoprotein cholesterol. Circulation, 121(11): 1347. http://doi.org/10.1161/CIRCULATIONAHA. 109.897330.

[9]. Saha, S., Graessler, J., Schwarz, P.E., Goettsch, C., Bornstein, S.R. & Kopprasch, S. 2012. Modified high-density lipoprotein modulates aldosterone release through scavenger receptors via extracellular signal-regulated kinase and janus kinase-dependent pathways. Molecular and Cellular Biochemistry, 366(1-2): 1-10.
https:// doi.org/10.07/s11010-012-1274-2

[10]. Rizzatti, V., Boschi, F., Pedrotti, M., Zaico, E., Sbarbati, A. & Zamboni, M. 2013. Lipid droplets characterization in adipocyte differentiated 3T3-L1 cells: size and optical density distribution. *European Journal of Histochemistry*, 57(3): 24. http://doi.org/10.4081/ejh.2013.e24.

[11]. Niemelä, S., Miettinen, S., Sarkanen, J.R., & Ashammakhi, N. 2008. Adipose tissue and adipocyte differentiation: molecular and cellular aspects and tissue engineering applications. Topics in Tissue Engineering, 4(1): 26.

[12]. William P. C. & Jaswinder K. S. 2015. TNF-α and adipocyte biology. *Federation of European Biochemical Societies,* 582(1): 117–131. doi: 10.1016/j.febslet.2007.11.051.

[13]. Özlem Sultan Aslantürk Submitted: 16 May 2017 Reviewed: 24 October 2017 Published: 20 December 2017 DOI: 10.5772/intechopen.71923 FROM THE EDITED VOLUME Genotoxicity - A Predictable Risk to Our Actual World