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Cytotoxic Effect of Tideglusib on Human Fibroblasts: An In Vitro Study

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Aim: This study evaluated the cytotoxic effect of different concentrations of Tideglusib as a GSK-3 inhibitor drug on human fibroblasts.

Materials and methods: Fibroblasts were cultivated and incubated under 5% CO2 and 95% humidity at 37oC for one day to achieve maturity and confluent growth. In the experimental group, cultures were subjected to different concentrations (500 nM/mL, 250 nM/mL, 125 nM/mL, 62.50 nM/mL, and 31.25 nM/mL and 15.60 nM/mL) of Tideglusib drug dissolved in Dimethyl sulfoxide (DMSO). In the positive control group, cell cultures were supplemented with equivalent volumes of DMSO solution. For negative control group, medium only was used without addition of Tideglusib or DMSO. For evaluation of cytotoxicity, Methyl Thiazol Tetrazolium (MTT) assay was used. Viability percentage was determined for each group and cytotoxicity responses were scored as: severe cytotoxic ≤30%, moderate cytotoxic 30%-60%, mild cytotoxic 60%-90%, and non-cytotoxic ≥90%. Data were statistically analyzed by One-way ANOVA followed by Tukey's post hoc test.

Results: Treatment with Tideglusib drug at the concentrations of 500, 250, 125, 62.50 nM/mL showed severe cytotoxic effects on fibroblasts and significantly decreased the percentage of cell viability when compared to the positive control ($P<0.001$). Treatment with 31.25 nM/mL and 15.60 nM/mL Tideglusib showed moderate and mild cytotoxic effects, respectively, and significantly decreased the percentage of cell viability when compared to the positive control ($P<0.001$).

Conclusion: Tideglusib showed a dose-dependent cytotoxic impact on fibroblast viability. Lower doses of Tideglusib, beginning at 31.25 nM/mL, resulted in cell viability rate of more than 50%.

Keywords: Cytotoxicity, GSK-3 inhibitor, Tideglusib, Fibroblasts, Viability.

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Introduction

Dental pulp vitality is regarded a significant element for protection against numerous external irritants. Microorganisms can penetrate the root canal system quickly through exposed dentinal tubules in the presence of necrotic pulp owing to lack of pulp defensive function.[1,2] Odontoblasts responsible for pulp protection by secretion of extracellular dentin matrix which protect the pulp against external stimuli.[3] However, teeth with vital pulps demonstrate greater resistance to bacterial invasion. Thus, keeping pulp vitality is a key aspect of the defensive system against bacterial invasion.[4] Various clinical situations may arise after the removal of decaying tooth structure, such as unintentional exposure of pulp tissue, which may jeopardize the life of the dental pulp.[5]

Direct pulp capping is a non-invasive and effective therapy that involves placing dental biocompatible material over exposed pulp tissue following odontoblastic layer injury.[6,7] This restoration is required to seal the exposed area and induce differentiation of dental pulp stem cells into odontoblast-like cells for reparative dentine production.[8] The capping material employed in direct capping processes has been identified as a key element in treatment effectiveness.

To enable adequate healing of exposed dental pulp, the pulp capping material must be bioactive, biocompatible, and antimicrobially active against a variety of microorganisms.[1,4,9] Different pulp capping materials have been studied in the literature, including calcium hydroxide, mineral trioxide aggregate, and other calcium silicate-based compounds.[1, 4, 10, 11] Various studies have shown that calcium hydroxide improves healing and reparative dentin production.[12,13] However, several additional limitations were demonstrated, including tunnel faults in the deposited dentinal bridge.[12] Other research recommended using mineral trioxide aggregate (MTA) because it has a superior sealing ability and improves dentinal bridge quality.[9,11,14] MTA disadvantages, such as lengthy setting time and probable discoloration, prompted the development of additional calcium silicate-based polymers, such as biodentine, to address these shortcomings and increase physiochemical qualities.[7, 8,14]

Tideglusib is a glycogen synthase kinase-3 (GSK-3) inhibitor that belongs to the thiadiazolidine family. It prevents inflammation and neurodegeneration.[15,16] Tideglusib is now being studied for clinical usage in the treatment of many neurodegenerative disorders, including Alzheimer's disease.[17] It activates stem cells by activating the Wnt/-cat signaling pathway, resulting in recovery. This pathway controls odontoblast differentiation and dental pulp stem cell proliferation.[18] It has been shown in the literature that applying the GSK-3 inhibitor Tideglusib directly to the region of pulp exposure can activate the Wnt/-cat signaling pathway, prompting stem cells to create reparative dentin and completely restoring injured dentin.[19,20]

The pulp capping materials should form a hermetic seal and promote wound healing.[1,4,9] Biocompatibility is also an important feature since these capping materials come into close touch with crucial pulp tissue for an extended period of time.[21] Cytotoxicity tests are routinely used to determine the biocompatibility of various materials when exposed to live cells. Various approaches have been proposed to evaluate the biocompatibility of dental materials. *In vitro* evaluation is the primary technique for this aim, and the Methyl Thiazol Tetrazolium (MTT) test is a colorimetric for assessment of cell viability.[22] MTT is taken into the mitochondria and converted to purple formazan by succinate dehydrogenase in live cells. The purple formazan is dissolved in an acidified solution before being added to a colored solution. The optical density of this

solution may be determined by measuring it at a certain wavelength. The cytotoxicity and cell viability of the substance may be determined by increasing the reduction of formazan and measuring optical density.[23] This study evaluated the cytotoxic effect of different concentrations of Tideglusib as a GSK-3 inhibitor drug on human fibroblasts.

Materials and methods

Ethical approval

This study was approved by the Ethical Committee at Faculty of Dentistry, Ain Shams University, Egypt (The approval number: 105-15/07/2020).

Study design

In the current study, Tideglusib drug powder and Dimethyl Sulfoxide solvent (DMSO) were obtained from Sigma Aldrich company, USA. The human fibroblasts cell line was obtained from VACSERA CO, Egypt. The cytotoxic effect of Tideglusib drug was evaluated at different concentrations after twenty-four hours on the WI-38 human fibroblasts cell line using MTT assay.

In the current study, human fibroblasts were treated with different concentrations of Tideglusib drug diluted and dissolved in DMSO solvent and served as the Tideglusib group. In the other group (Positive control), human fibroblasts were treated with the same amount of DMSO solvent only without addition of Tideglusib drug to indicate any potential effects of DMSO solvent on cells viability. Human fibroblasts were incubated in culture media without any additional treatment to determine the base line of cell viability, served as negative control group.

Cell culture

Preparation of cell cultures: basic standard protocols were followed for maintaining cell cultures. Fibroblasts were routinely cultivated in Dulbecco's Modified eagle's Medium (DMEM) supplied with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 µg/mL of penicillin. The cultures were incubated under 5% CO_2 in 95% humidity (Jouan- France) to achieve maturity and growth needed for experimentation. Fibroblasts were detached by trypsinization and plated in 96- cluster well culture plate at concentration of $1x10^4$ cells in every well. Each well contained 100µL of cell suspension and incubated at 37ºC for 24 hours under 5% CO_2.[23]

Cytotoxicity assay (MTT assay)

The appropriate amount of Tideglusib drug powder was calculated according to its molecular weight (334.39 g/mol) and its solubility in DMSO solvent (>15mg/mL) and dissolved in DMSO solution to reach the desired concentration. The calculated Tideglusib amount was dissolved in 10 mL of DMSO solvent to prepare a 0.5 mM which is equal to 500 nM/mL. The solution was thoroughly mixed using a vortex mixer to ensure uniform distribution and this concentration served as the starting concentration.

Further descending dilutions were prepared to reach the desired concentrations (250 nM/mL, 125 nM/mL, 62.50 nM/mL, 31.25 nM/mL and 15.60 nM/mL). The starting concentration 500 nM/mL was twofold serially diluted in DEME medium containing 2% FBS, the serially dilutions were dispensed to six columns (each column different dilution) as 0.1mL/well of each diluted solution.

Preparation of controls: in positive control group, cell cultures were supplemented with equivalent volumes of DMSO solution. DMSO as solvent was processed as the Tideglusib to account for any cytotoxic effects of DMSO on fibroblast viability. In negative control group (medium only containing fibroblasts), cell cultures were supplemented with DMEM medium

containing 2% FBS without addition of Tideglusib or DMSO and used for assessment of the baseline of cell viability. The negative control group determined the average of optical density for calculation of viability percentage in Tideglusib and positive control groups.

Fibroblasts were treated and incubated with the serially diluted different Tideglusib concentrations and positive control group for 24 hours at 37°C in CO_2 condition. Dead cells were removed out using phosphate buffer saline (PBS). MTT stain was added as 0.05 mL per well at a concentration of 0.5 mg/mL. Four hours later, viable cells metabolized the MTT dye to form insoluble formazan crystals. MTT- formazan complex crystals were dissolved using DMSO 0.05 mL/well, after 15 min to ensure complete dissolution of formazan crystals. Absorbance of each well at a wavelength of 570 nm using an ELISA plate reader spectrophotometer (ELX-800, Biotek, USA) was recorded.[24]

For Tideglusib and positive control groups, the following formula determined the cell viability percentage [Mean OD of treatment group/Mean OD of negative control group] × 100% [1,2]. Cytotoxicity responses were scored as: severe cytotoxic ≤30%, moderate cytotoxic 30%-60%, mild cytotoxic 60%-90% and non- cytotoxic ≥90%.[23]

Statistical evaluation

The data were analyzed using the Statistical Package for the Social Sciences software, version 20 analysis (IBM® SPSS). Descriptive analysis of the data included cell viability percentage in terms of mean ± standard deviation values were calculated for each group and for each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way ANOVA followed by Tukey's post hoc test was used to compare between the mean percentages of cell viability between all groups at the same time interval. The significance level was set at $P \leq 0.05$.

Results

Cytotoxicity assay results

Differences in the mean viability percentages of all groups are shown in table (1) and figures (1, 2, and 3). Treatment with Tideglusib drug at the concentrations of 500, 250, 125, 62.50 nM/mL showed severe cytotoxic effects on fibroblasts and significantly decreased the percentage of cell viability when compared to the positive control (P<0.001). Treatment with 31.25 nM/mL and 15.60 nM/mL Tideglusib showed moderate and mild cytotoxic effects, respectively, and significantly decreased the percentage of cell viability when compared to the positive control (P<0.001).

There were statistically significant differences in cell viability between Tideglusib and DMSO at all concentrations (P <0.001). Also, there were statistically significant differences in cell viability between the different used concentrations of Tideglusib (P <0.001) except between 250 nM/mL and 125 nM/mL.

The highest viability percentage was scored with Tideglusib concentration of 31.25 and 15.60 nM/mL.

Table (1): The mean and standard deviation (SD) values of cell viability percentages in both groups.

Concentrations	Tideglusib group		Positive control group (DMSO)		*P*-value
	Mean	SD	Mean	SD	
Conc. 500	12.63 ^{cB}	0.62	20.41 ^{fA}	1.20	<0.001*
Conc. 250	18.88 ^{dB}	0.62	34.26 ^{eA}	0.85	<0.001*
Conc. 125	20.75 ^{dB}	0.93	45.26 ^{dA}	0.69	<0.001*
Conc. 62.50	23.78 ^{cB}	0.93	63.19 ^{cA}	1.73	<0.001*
Conc. 31.25	53.78 ^{bB}	1.32	72.27 ^{bA}	1.73	<0.001*
Conc. 15.60	74.44 ^{aB}	2.32	86.35 ^{aA}	3.39	0.001*
P-value	<0.001*		<0.001*		

Means with different lower-case superscripts in the same column indicate significant difference. Means with different upper-case superscripts in the same row indicate significant difference. *: Significant at P<0.05.

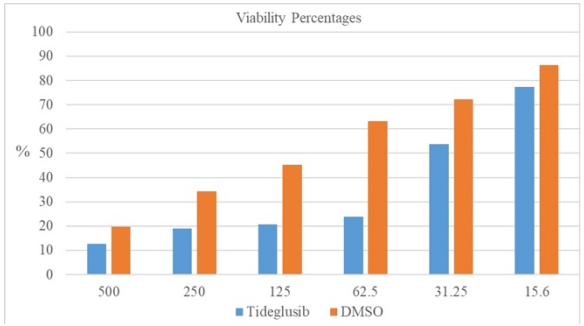

Figure (1): Bar chart representing viability percentages across both groups

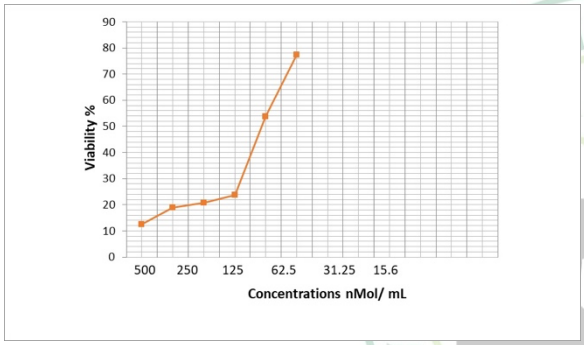

Figure (2): Viability percentages across different Tideglusib concentrations.

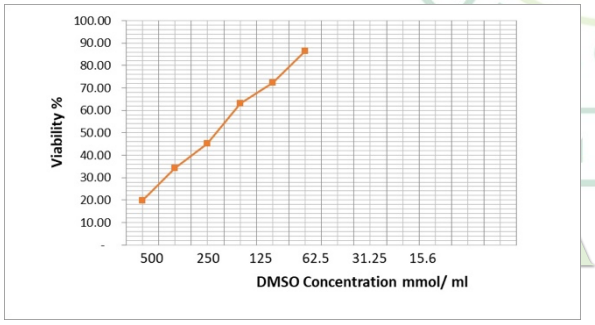

Figure (3): Viability percentages across different DMSO concentrations.

Discussion

There are several variations between trials, notably in terms of Tideglusib concentrations and assessment times.[21,25] As a result, the major goal of the current study was to evaluate the effects of Tideglusib on human fibroblasts, with a special emphasis on cytotoxicity evaluation. This study found that Tideglusib had dose-dependent cytotoxic effects on fibroblasts, with greater doses resulting in a lower cell viability %. The findings of this study may focus future research on developing new distinct treatment techniques for boosting dentin-pulp complex regeneration and improving clinical outcomes in dental pulp therapy.

The use of fibroblasts as the principal cell type in this study was intentional since they are the most common cell type seen in connective tissue, including pulp tissue and periodontal ligament. Stem cells within the dental pulp can differentiate into fibroblasts and odontoblast like cells to produce dentin pulp complex like tissue.[26] Studying fibroblast responses to Tideglusib may give light on the drug's potential impacts on dentin-pulp complex regeneration and periodontal tissue health.[27] Furthermore, the use of fibroblasts in this study aligns with the goal of assessing Tideglusib biocompatibility and possible functions in promoting or inhibiting tissue regeneration in the oral environment.

In vitro cell studies are widely used to examine the cytotoxic effect of novel dental materials. These investigations usually include determining the percentage of cell viability at the conclusion of a set experimental period. The choice of viability tests is critical for correctly analyzing drugs' effects on cell viability and cytotoxicity.[28] Different assays are available, including tetrazolium reduction assays such as MTT, XTT, MTS, and WST1, which are widely used because to their dependability and efficacy. The MTT test, in example, is a popular choice due to its simplicity, low cost, speed, and accuracy.[22,29,30]

This test depends on live cells reducing MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to create insoluble formazan crystals that can be measured spectrophotometrically.[16,29,30] As a result, we chose the MTT test for assessing cell viability in the current study because of its accuracy, simplicity, cost, speed, and efficacy. Pulp capping agents should

preferably have low cytotoxicity and cause minimum or no inflammatory responses when in contact with pulp tissue.[31] According to Neves and Sharpe[32], Tideglusib may provide additional benefits beyond its usual role as a GSK-3 inhibitor because it has the ability to increase stem cells within the tooth pulp by encouraging their proliferation and differentiation.

In the current investigation, substantial declines in fibroblast viability % were seen after 24 hours of Tideglusib administration at increasing doses. These data indicate that Tideglusib had a dose-dependent cytotoxic impact on cell viability, with lower dosages resulting in larger enhancement.
Several studies in the literature have shown that activating the Wnt signaling pathway promotes fibroblast activation, proliferation, and differentiation into myofibroblasts via inducing fibrosis.[33] Myofibroblasts are contractile cells that aid in wound healing and fibrosis. This stimulation can occur through the canonical Wnt/β-catenin pathway or non-canonical proliferative pathways.[34,35] Akhmetshina et al[36] found that Wnt activation boosted lung fibroblast proliferation and differentiation, as well as the number of fibroblasts.

Wnt activation and GSK-3 inhibition reduced TGF-β1 expression in gingival fibroblasts, inhibiting gingival development, contrary to prior research.[37] After 24 hours of incubation, Tideglusib did not stimulate a proliferative response in human fibroblasts. Instead, we detected a significant reduction in cell viability, particularly at higher Tideglusib doses compared to the control group. Our findings are consistent with those of Oncu et al[25] who studied the cytotoxic impact of various Tideglusib doses (200 nM/mL, 100 nM/mL, and 50 nM/mL) on fibroblasts obtained from periodontal ligament and gingival tissues. They concluded that increasing concentrations of Tideglusib had no proliferative impact on fibroblasts, and that cytotoxicity rose dramatically after 24 hours of high concentrations. They also discovered that the lower concentration of Tideglusib begins at 50 nM/mL to maintain cell viability. Similarly, Neves et al[19] used MTT tests to determine the cytotoxic effect of various Tideglusib doses on 17IA4 mouse dental pulp cell lines. They discovered that Tideglusib at 200 and 100 nM/mL had the lowest viability percentage when compared to 50 nM/mL.

The current investigation found that the viability percentage remained over 50% at Tideglusib values of 31.25 nM/mL and 15.60 nM/mL. These findings from our study support those of previous authors.[16] They found that Tideglusib had the maximum viability percentage on rat dental pulp cells at a dosage of 34 nM/mL. This uniformity across investigations lends credence to the observed effects of Tideglusib on cell viability, increasing the dependability of our findings. It implies that doses of 15-50 nM/mL may be particularly helpful in preserving cell viability at high levels while also exhibiting favorable biological effects. This agreement emphasizes the importance of repeatability in scientific research, improves knowledge of the Tideglusib dose-cytotoxicity relationship, and explores prospective uses in direct pulp capping.

The choice of assessment time in our study may be seen as a restriction since it may impact the observed results. Hana et al[38] did a research to examine the cytotoxicity of various Tideglusib doses on dental pulp stem cells over a longer period of time, 72 hours. Interestingly, they discovered that Tideglusib improved cell viability after 72 hours at 100 nM/mL Tideglusib concentration compared to 24 hours. They found that the NANOG gene was highly expressed in the Tideglusib group, indicating that it had a good effect on the stemness of human dental pulp stem cells.

The current study's findings have the potential to enlighten future research and clinicians on the use of Tideglusib in therapeutic treatments targeted at stimulating tissue regeneration or treating pathological diseases. However, it may be necessary to investigate the cytotoxicity of Tideglusib on human fibroblasts over extended periods of time.

Conclusion

Higher Tideglusib concentrations (500-62.5 nM/mL) reduce fibroblast viability. Tideglusib can be administered safely at doses as low as 31.25 nM/mL, with a viability rate of more than 50%.

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