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In Vitro Induction of Apoptosis in Human Lymphocytes By Stx1 From E. Coli O157:H7

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ABSTRACT

Background: Shiga toxin 1 (Stx1), produced by Escherichia coli O157:H7 and related strains, is a potent cytotoxin implicated in severe clinical conditions such as hemolytic uremic syndrome (HUS), acute kidney injury, and chronic urinary tract infections. While its mechanisms of protein synthesis inhibition and apoptosis induction are well characterized in epithelial and endothelial cells, its effects on immune cells particularly human lymphocytes remain underexplored. This study aimed to investigate the apoptotic response of human peripheral blood lymphocytes to Stx1 exposure in vitro to better understand the immunomodulatory potential of the toxin. Lymphocytes were isolated from the peripheral blood of healthy donors using density gradient centrifugation. Cells were treated with 5 µL of Stx1 toxin at three concentrations (0.25 ng/mL, 0.5 ng/mL, and 1 ng/mL) and incubated with 5 µL of acridine orange/ethidium bromide staining solution at four-time intervals (0 h, 4 h, 12 h, and 24 h). A control group of untreated cells was included at each time point. Apoptotic changes were assessed via fluorescence microscopy based on nuclear staining patterns. A substantial increase in apoptosis was observed in Stx1-treated lymphocytes compared to controls. The effect was both dose- and time-dependent, with the highest apoptotic rate noted at 1 ng/mL after 24 h. Control groups showed minimal baseline apoptosis. Stx1 exerts a strong pro-apoptotic effect on human lymphocytes in vitro, suggesting a potential role in immune suppression and pathogenesis during E. coli O157:H7 infections. These findings underscore the importance of considering lymphocyte impairment in the clinical course of HUS and related conditions.

KEYWORDS

Biosynthesis, caspase, centrifugation, clinical, protein, time

CITATION

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1. Introduction

A pathogenic bacterial strain, *Escherichia coli* O157:H7, produces potent toxins, most notably Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) (Bova *et al.*, 2023). Primarily associated with outbreaks of foodborne illness, these toxins can lead to serious clinical conditions such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) a life-threatening disease characterized by renal failure, thrombocytopenia, and hemolytic anemia (O'Brien and Holmes, 1987). Stx1, in particular, is a powerful cytotoxin that induces cell injury and death by inhibiting protein synthesis in eukaryotic cells (Johannes and Römer, 2010). While the cytotoxic effects of Shiga toxins (Stxs) on epithelial and endothelial cells are well documented, their impact on immune cells particularly human lymphocytes remains poorly understood (Kurohane *et al.*, 2014). Lymphocytes, including T and B cells, are critical components of the adaptive immune system, playing central roles in defending the body against infection. The ability of Stxs to influence lymphocyte survival and function may have important implications for the immune response during *E. coli* O157:H7 infection (Jamshidi *et al.*, 2015). Apoptosis is a crucial process in immune regulation (Luvisetto, 2020), ensuring the removal of infected, damaged, or non-functional cells without triggering an inflammatory response (Tesh and O'brien, 1991). Disruption of apoptosis can lead to immune dysfunction, contributing to persistent infection, autoimmunity, or immunodeficiency (Creagh *et al.*, 2003). Although recent studies suggest that Stx1 can induce apoptosis in various cell types, including epithelial cells, its effects on lymphocytes have not been thoroughly investigated (Jones *et al.*, 2000). In this study, we aim to examine the induction of apoptosis in human lymphocytes *in vitro* by the Stx1 protein derived from *E. coli* O157:H7. We specifically investigate how Stx1 affects lymphocyte viability, the molecular pathways involved, and the role of apoptotic regulators such as Bax/Bcl-2 and caspase activation. Gaining insight into how Stx impacts lymphocyte apoptosis may help elucidate the broader immunological consequences of *E. coli* O157:H7 infection and its contribution to immune dysfunction.

2. Materials and Methods

Stx1 was obtained from *E. coli* O157:H7 strains using approved toxin extraction protocols, following the established method described in (Hames, 1998). The toxin was concentrated and stored at −80 °C until use. Human lymphocytes were used in this study to assess the effect of the toxin on apoptosis induction in blood cells, following the procedures outlined in (Salvioli *et al.*, 1997). Ten milliliters of blood were collected from healthy volunteers after obtaining informed written consent. Ethical approval for the study was granted by the Ethics Committee of Wahj Al-Dana Center for Training and Scientific Development (Approval No: Wahj-Al DNA-IRB-2025-009).

The collected blood samples were treated with the anticoagulant heparin and refrigerated until further processing. Lymphocytes were isolated by density gradient centrifugation using lymphocyte separation medium (LSM). Briefly, the blood was diluted 1:1 with freshly prepared phosphate-buffered saline (PBS), pH 7.2. The diluted blood was carefully layered over the LSM in centrifuge tubes and centrifuged at 400 × g for 35 minutes (Boyum, 1986; Favour, 1964). Following centrifugation, three distinct layers formed: an upper plasma layer, a middle layer containing lymphocytes along with some platelets and monocytes, and a bottom LSM layer. The lymphocyte layer was carefully collected using a micropipette. The isolated lymphocytes were washed three times with PBS by centrifugation at 250 × g for 10 minutes each to remove residual platelets, LSM, and plasma.

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2.1. Preparation of the AO/EB Solution:

According to (Haris, 1969; Ting and Morris, 1971), the following preparatory steps were followed:

Ten microliters of acridine orange were mixed with ten microliters of ethidium bromide to obtain the acridine orange/ethidium bromide (AO/EB) solution. Then, 25 microliters of the previously prepared lymphocyte suspension were mixed with 1 microliter of the AO/EB solution. Each sample was immediately examined and evaluated by placing 10 µL of the stained lymphocyte suspension onto a microscope slide and covering it with a cover glass. At least 300 cells were examined using a fluorescence microscope equipped with an imaging system. Two filters were used for each image capture: FITC for green detection and Texas Red for red detection. The two images were combined to assess the apoptotic or necrotic status of the cells.

2.2. Sample and Slide Preparation:

Toxin dilutions (1 ng/mL, 0.5 ng/mL, and 0.25 ng/mL) were prepared by mixing 25 µL of cells with 5 µL of toxin and 5 µL of AO/EB staining solution. The preparation was carried out by mixing, and the mixture was incubated for different durations (0 h, 4 h, 12 h, and 24 h). After incubation, the sample was placed on a slide and stained at room temperature. The viability of live cells (red fluorescent region) and dead cells (green region) was analyzed using a fluorometer and the FITC channel.

The percentage of apoptotic cells was calculated using the following equations (Lee *et al.*, 2005):

Viable cells (%) = (Red count / [Red count + Green count]) × 100

Apoptosis (%) = 100 − Viable cells (%).

• **Red cells** represent live cells or cells that have not undergone apoptosis.

• **Green cells** represent cells that have undergone apoptosis or are marked as dead.

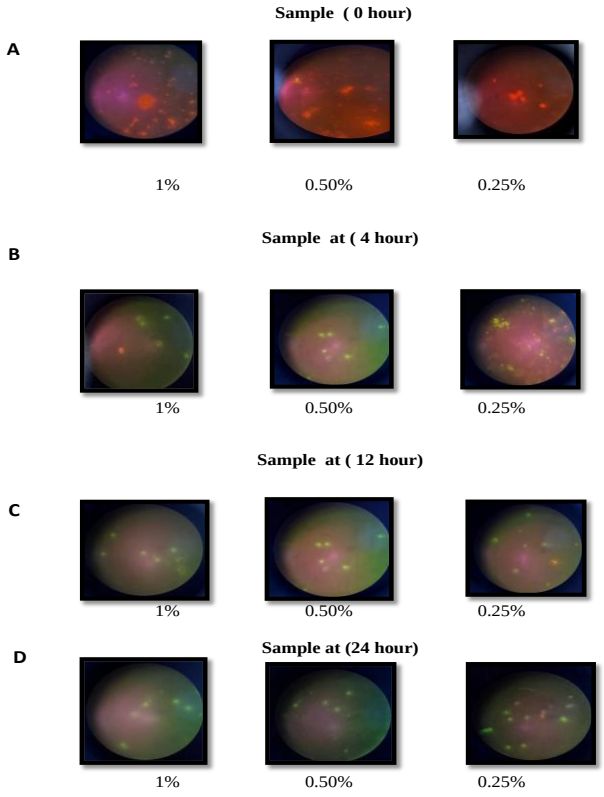
This study was designed as an *in-vitro* experimental investigation to evaluate the apoptotic effect of Stx1 on human peripheral blood lymphocytes. Peripheral blood samples were obtained from healthy adult volunteers following informed consent, and ethical approval was secured from the Wahj Al-Dana Center for Training and Scientific Development (Approval No: Wahj-Al DNA-IRB-2025-001). The study included four experimental groups: three treatment groups exposed to different concentrations of Stx1 (0.25 ng/mL, 0.5 ng/mL, and 1 ng/mL) and one untreated control group. Each group was assessed at four-time intervals (0 h, 4 h, 12 h, and 24 h). Lymphocyte apoptosis was quantified using AO/EB dual staining followed by fluorescence microscopy. All experiments were performed in triplicate to ensure reproducibility.

The primary outcome was the percentage of apoptotic cells at each concentration and time point, providing insight into both the dose- and time-dependent effects of Stx1. Statistical comparisons were made between control and treated groups to evaluate the significance of observed effects.

3. Results and Discussions

The results of the study shown in Figure 1-A indicate that the toxin had no effect at time zero across all tested concentrations. However, Figure 1-B shows a gradual increase in the toxin's effect on the cells over time at a concentration of 0.25 ng/mL. As shown in Tables 1 and 2, the percentage of live cells decreases to 95%, 86%, 60%, and 55%, while the corresponding apoptosis rate increases to 5%, 14%, 40%, and 45%, respectively, over time.

Figure 1. Stages of programmed apoptosis at the tested toxin concentrations and time intervals.



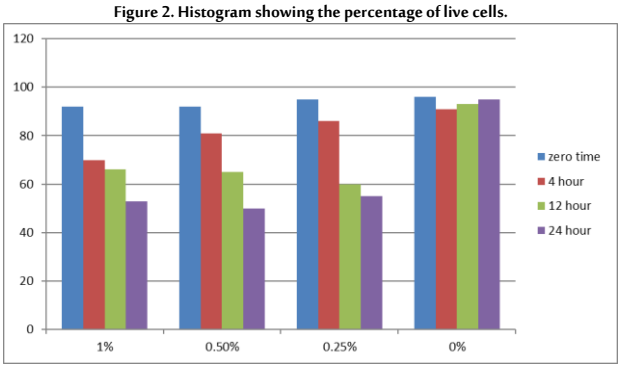
In Figure 1-C, the toxin continues to exert a stronger effect on the cells, with the percentage of live cells decreasing further to 92%, 81%, 65%, and 50%, and the apoptosis rate increasing to 8%, 19%, 35%, and 50%, respectively, at a toxin concentration of 0.50 ng/mL, as shown in Tables 1 and 2 for the same time intervals. In Figure 1-D, with the use of a toxin concentration of 1 ng/mL, the results of the current study show a decrease in the percentage of live cells to 92%, 70%, 63%, and 47%, and a corresponding increase in the apoptosis rate to 8%, 30%, 37%, and 53% over time, as also presented in Tables 1 and 2.

According to the above equation, the results were as follows:

Table 1. Percentage of viable (live) lymphocytes.

Toxin concentration (ng/mL)	0h Viability (%)	4h Viability (%)	12h Viability (%)	24h Viability (%)
1.00	92%	70%	63%	47%
0.50	92%	81%	65%	50%
0.25	95%	86%	60%	55%
0.00	97%	95%	95%	95%

Figure 2. Histogram showing the percentage of live cells.



Apoptosis (%) = 100% − Viability (%)

Table 2. Percentage of apoptotic lymphocytes.

Toxin concentration (ng/mL)	0h Apoptosis (%)	4h Apoptosis (%)	12h Apoptosis (%)	24h Apoptosis (%)
1.00	8%	30%	37%	53%
0.50	8%	19%	35%	50%
0.25	5%	14%	40%	45%
0.00	3%	5%	5%	5%

Figure 3. Histogram shows apoptosis percentages.

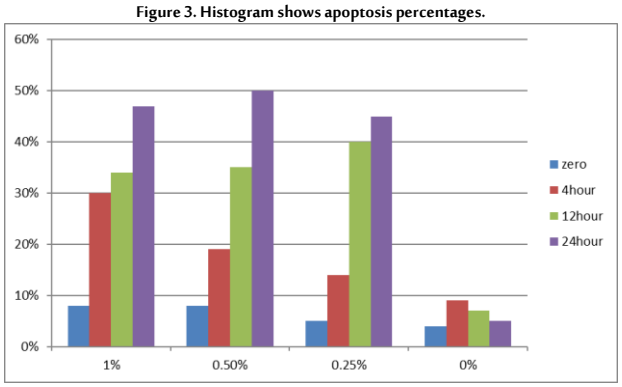
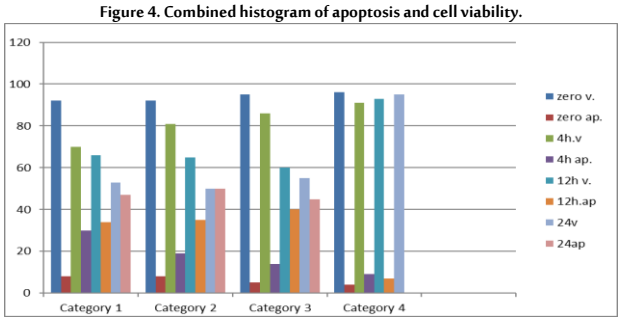


Table 3. Combined percentage of viable and apoptotic cells at each concentration and time point.

Toxin concentration (ng/mL)	0h Viability (%)	0h Apoptosis (%)	4h Viability (%)	4h Apoptosis (%)	12h Viability (%)	12h Apoptosis (%)	24h Viability (%)	24h Apoptosis (%)
1.00	92%	8%	70%	30%	63%	37%	47%	53%
0.50	92%	8%	81%	19%	65%	35%	50%	50%
0.25	95%	5%	86%	14%	60%	40%	55%	45%
0.00	97%	3%	95%	5%	95%	5%	95%	5%

Figure 4. Combined histogram of apoptosis and cell viability.



From the results shown in Tables 1, 2, and 3, we note that Stx1 extracted from *E. coli* O157:H7 has a substantial toxic effect on human lymphocytes, leading to increased apoptosis (programmed cell death) in a dose- and time-dependent manner. This finding aligns with what was reported in (Lee *et al.*, 2005). The highest rate of programmed cell death in this experiment was observed at a toxin concentration of 1% after 24 h, reaching 53%.

3.1. Effects of Toxin Concentration:

High concentration (1%): The results show that the highest concentration of toxin (1%) caused the strongest toxic effect on lymphocytes, with cell viability decreasing to 47% and apoptosis increasing to 53% after 24 h of exposure. This indicates that 1% of Stx1 induces a strong effect on lymphocytes, leading to substantial cell death over a prolonged period. It suggests that the toxin disrupts cellular processes irreparably at higher concentrations, likely triggering apoptosis pathways that ultimately lead to lymphocyte loss. According to research conducted by (Lee *et al.*, 2005), Stxs may induce monocyte apoptosis through a novel mechanism that is independent of death receptors and dependent on caspase-8.

Moderate concentration (0.50%): At a concentration of 0.50%, the lymphocytes were less affected than at 1%, with 50% cell viability and 50% apoptosis. This suggests that even at moderate concentrations, Stx1 has a substantial apoptotic effect, though with a less pronounced impact compared to higher concentrations. It also indicates that the toxin progressively causes irreversible damage to lymphocytes as the concentration increases.

Low concentration (0.25%): Even at the lowest concentration (0.25%), about 45% of the cells underwent apoptosis after 24 h, indicating that the toxin has a considerable effect even at low doses. This supports the hypothesis that Stx can effectively induce apoptosis in lymphocytes, even at lower concentrations.

3.2. Effect Based on Time:

Zero time (before toxin exposure): At time zero, cell viability was high (ranging from 92% to 97%), indicating that the lymphocytes were healthy and unaffected prior to toxin exposure. Apoptosis levels were also low (3%–8%), suggesting that the cells were not undergoing spontaneous apoptosis before treatment. After 24 h: At the 24-h mark, apoptosis reached its highest levels (ranging from 45% to 53%), and cell viability declined substantially (from 47% to 55%). This demonstrates that the toxin causes progressive damage to lymphocytes, leading to cell death with prolonged exposure. It is important to mention that a study by (Kojio *et al.*, 2000) reported that caspase-3 activity peaked at 5 h after exposure of THP-1 cells to Stx1, while Stx1-induced mortality in THP-1 cells began at 4 h (Harrison *et al.*, 2005). The activities of caspase-3, -8, and -9 were measured from 0 h to 24 h following toxin treatment, and it was found that all caspase activities reached their peak levels after 8 h. These activities appeared to return to basal levels during the remaining 16 h of the experiment. In all toxin concentrations used in the current study, there is a directly proportional relationship with time, meaning that as the exposure time increases at a constant toxin concentration, the rate of cellular apoptosis also increases. This indicates that the toxin's effect intensifies over time, leading to a gradual induction of apoptosis with prolonged exposure. In other words, time plays a key role in enhancing the toxin's impact on the cells, as the percentage of cells undergoing programmed cell death (apoptosis) increases with the duration of toxin exposure. HUS also results in apoptotic cell death in human tissues (Karpman *et al.*, 1998). The mechanisms by which Stxs induce apoptosis are not yet fully characterized, although cell death caused by Stxs may be critical in the pathogenesis of hemorrhagic colitis and systemic vascular complications. Numerous studies have been conducted in Basra Governorate on *E. coli*, including (Albadery *et al.*, 2023; Hardany *et al.*, 2020), which demonstrated that monocytic THP-1 cells are susceptible to the cytotoxic action of Stxs, with a 50% cytotoxic dose of approximately 14 pg/mL (Ramegowda and Tesh, 1996). The findings of the present study, which demonstrate a dose- and time-dependent induction of apoptosis in human lymphocytes upon exposure to Stx1, align with recent evidence on the cytotoxic effects of Stxs. Notably, a comprehensive review by Wang *et al.* (2024) emphasized the immunopathological roles of Stx1 and Stx2, noting their ability to modulate immune cell survival and trigger apoptosis in host immune cells, including lymphocytes. This supports our observation of a peak apoptosis rate of 53% at 1 ng/mL concentration after 24 h of exposure, highlighting the immunosuppressive potential of Stx1 during *E. coli* O157:H7 infections (Wang *et al.*, 2024). Furthermore, a 2025 publication in the *Journal of Cancer Prevention* discussed the pathogenic mechanisms of Shiga toxin-producing *Escherichia coli* (STEC) strains and their contribution to immune dysregulation. The study underlined the ability of these toxins to interfere with T-cell responses and promote programmed cell death, reinforcing our findings that lymphocytes are direct and substantial targets of Stx1 toxicity (Babolhavaeji *et al.*, 2024). These recent studies collectively support the notion that, beyond its classical vascular and renal targets, Stx1 has broader implications for host immune competence. Our findings contribute

to this growing body of knowledge by providing empirical evidence that peripheral blood lymphocytes undergo apoptosis in response to Stx1 exposure, potentially compromising the adaptive immune response during STEC infections.

4. Conclusions

These results confirm that Stx1, extracted from *E. coli* O157:H7, induces apoptosis in human lymphocytes in a dose- and time-dependent manner. The highest concentration (1%) resulted in the most severe cell death, with apoptosis rates exceeding 50% after 24 h of exposure. Even at lower concentrations (0.50% and 0.25%), the toxin caused a substantial degree of cell death, albeit to a lesser extent. These findings suggest that Stx plays a critical role in immune system dysfunction and may contribute to disease manifestations such as HUS in patients infected with STEC.

Data Availability Statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no competing interests.

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