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Mechanisms of Action of Toxins Released by *Clostridium perfringens*

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ABSTRACT

Clostridium perfringens, a rod-shaped anaerobe, is a Gram-positive bacterium that causes foodborne diseases. Its generation time is less than ten minutes and it can divide at 45°C. This aerotolerant bacterium has some toxigenic types (A, B, C, D, and E) that can cause diseases in human beings. Two of its newly discovered toxin types are F and G. Histotoxic, neurological, and intestinal illnesses in both people and animals are instigated by *C. perfringens* due to its wide range of protein toxins. Alpha or CPA, beta or CPB, epsilon or ETX, iota or ITX, and enterotoxin or CPE are the primary toxins that contribute toward diseases. CPA is the primary pathogenicity factor in gas poisoning in human beings, despite its limited and debatable involvement in animal illnesses. Necrotizing intestinal inflammation and enterotoxaemia in infants of various vertebrate species, particularly humans, are caused by CPB. Some other types cause illnesses in livestock. Necrotic and apoptotic traits are present in the molecular pathways of cell damage linked to *C. perfringens* toxins.

Keywords: apoptosis, cell killing, *Clostridium perfringens*, necrosis, pathways, toxins

1. INTRODUCTION

Clostridium perfringens is an anaerobic microorganism that inhabits the intestines of animals and human beings and causes gastrointestinal infection. This infection can enter into the general circulation and may cause enterotoxaemia [1]. This diverse group of Gram-positive bacteria causes various diseases including clostridial myonecrosis, botulism, enteritis necroticans, food poisoning, and tetanus [2]. This pathogen has a ubiquitous nature. It forms spores that cause problems in the food industry and the pathogen is considered as major factor in epidemiological outbreaks. Histotoxic and enterotoxic diseases are mediated by the generation of potent protein toxins [3]. Infections caused by this pathogen depend upon the possession of toxin plasmids as the toxinotyping classification of this pathogen shows its various types. These toxin plasmids cause specific disease syndromes [4].

2. HISTORICAL BACKGROUND

C. perfringens was first identified at the end of the 19th century when a case was reported in which the patient died with aortic aneurism. Since then, many microorganisms have been isolated from foods that cause foodborne illnesses [5].

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Firstly, the above pathogen was identified as *Bacillus aerogenes capsulatus.* Later on, it was labelled as *Bacillus welchii*. Finally, it was labelled as *C. perfringens*. In Latin it means "burst through" [6]. It was observed that if a large number of spores are ingested, it produces enterotoxin that was isolated in 1970s. Extensive studies have been conducted on the toxin types of this specie and this survey provides information regarding its role in foodborne illnesses [7]. Selective media was used for the rapid growth of this pathogen at high temperature. It was observed that it has the ability to resist specific antibiotics [8].

3. FEATURES OF THE RESERVOIR

Clostridium constitutes encapsulated, spore producing, non-motile, and anaerobic Gram-positive rods which lack flagella. Colonies may be large, round, opaque, and shiny, exhibiting double zone hemolysis by alpha toxin release [9]. It is also used as an indicator in Europe in water resources [10].

4. OCCURRENCE

4.1 Feces

C. perfringens is found in animal as well as human feces. This is because it is present in intestine where it causes infections. This pathogen has been observed with variations over time. In healthy persons, it is detected in a normal amount, that is, <105 cfu/g of feces. However, the affected patient has >106 cfu/g of *Clostridium* in their feces [11]. Further, a higher level of this pathogen is found in adults as well as in newborns [12].

4.2 Food

After many surveys, it was concluded that it is an etiological agent of food poisoning. It is also found in raw meat as well as in frozen items [13]. The epidemiological outbreak of foodborne diseases is linked with poultry items in particular and with all other food items in general.

5. CLASSIFICATION AND TOXINOTYPES

C. perfringens belongs to the kingdom Bacteria, phylum Firmicutes, order Clostridiales, family *Clostridiaceae,* and genus *Clostridium* [14].

Different strains of *C. perfringens* have been identified which produce different toxins. Strain to strain variations show that there are more than one toxin genes on the plasmid [15]. Keeping all these conditions in view, *C. perfringens* are classified in this study into 5 toxinotypes, namely A, B, C, D, and E [16]. Further, F and G toxinotypes [17] are newly purposed strains discovered through experiments. All strains generate alpha toxin. Moreover, they also generate some other toxins shown in the table below (Table 1).

Table 1. Toxin Based Classification of *C. perfringens* [16]

Type	Toxins					
	CPA	CPB	CPE	ETX	ITX	NetB
A	+	-	-	-	-	-
B	+	+	-	+	-	-
C	+	+	+/-	-	-	-
D	+	-	+/-	+	-	-
E	+	-	+/-	-	+	-
F	+	-	-	-	-	-
G	+	-	-	-	-	+

C. perfringens alpha, *C. perfringens* beta, *C. perfringens* enterotoxin, *C. perfringens* epsilon toxin and *C. perfringens* iota toxin

are abbreviated as CPA, CPB, CPE, ETX and ITX respectively.

The above classification scheme may not serve epidemiological purposes. Currently, new and very different toxins have been discovered through experiments that cause very different illness. Hence, in the new and updated classification scheme of toxins, two more toxinotypes namely F and G have been included [18].

5.1. Type A

This is one of the most common toxins that cause food poisoning in the US. Type A *C. perfringens* produces CPE. Occasionally, it is linked with non-food borne illnesses as well, such as antibiotic related illnesses and sporadic diarrhea [19]. Once ingested, it causes illness. It influences spore resistance through the acid soluble protein generated by infection. It produces alpha toxin only [20].

5.2 Type B

This type is majorly detected in goats, calves, and foals, where it causes a disease known as lamb dysentery. It gets transferred directly through environment. After entry, it starts to divide. Then, it produces toxins (α, β, and ε toxins) [21].

5.3 Type C

This type of *C. perfringens* has been detected in both humans and livestock including horses, sheep, and pigs. Type C produces alpha, beta, and CPE toxins. Enteritis necroticans or the inflammation of intestine is considered as its major manifestation with bloody stool and abdominal pain. Death may also occur within 48 hours [22].

5.4 Type D

It mostly occurs in animals with acute, sub- acute, and chronic phases. Infection leads to neurological and respiratory variations. It produces alpha, epsilon, and CPE toxins [23].

5.5 Type E

Type E produces iota toxin. It is observed in rabbits, cattle, and lambs. It carries silent CPE sequences and also produces CPA toxin[24].

5.6 Type F

It contains the genes of both CPA and CPE toxins (released only during the sporulation process), causing food poisoning and diarrhea. Accumulation of fluid is the major manifestation. In experiments on rabbits, it showed mucosal damage [16].

5.7 Type G

It releases alpha toxin as well as Net B toxin, causing diseases in chickens. Koch's postulates were implemented experimentally and proved that it causes necrotic enteritis. Occasionally, mutation in genes leads to lesions formation. It is used in the epidemiological detection procedure [16].

6. TOXINS PRODUCED BY *C. PERFRINGENS*

Cellular intervention of toxins produced by *C. perfringens* begins with the toxic substance binding to a receptor located on the plasma membrane of the host cell. This is accompanied by the stimulation of cellular events and an assortment of cytopathic consequences, all of which ultimately cause cell death [1].

6.1. *C. perfringens* Enterotoxin (CPE)

All toxins produced by this pathogen interact with cell membrane. Only the iota toxin produced by type E doesn't interact at all with the cell membrane. After interaction, it forms pores and hydrolytic

enzymes are secreted. The production of toxins only takes place during sporulation. The gene for expressing CPE can be found on the same locus of chromosome in strains associated with food poisoning [25].

6.1.1. General Mechanism of Action. A 'compact molecule' of about 90 kDa forms when CPE first binds to claudin channels [26]. A pre-pore is formed on the surface of the membrane as a result of the association of numerous (about 6) tiny clusters [27]. The end product, known as CH-1, is a 'big structure' of around 450 kDa that includes the CPE hexamer, as well as receptor and non-receptor claudins [26]. When beta-hairpin are assembled into a beta-barrel and quickly inserted into the target biological membrane, an ionically charged hole begins to develop [28].

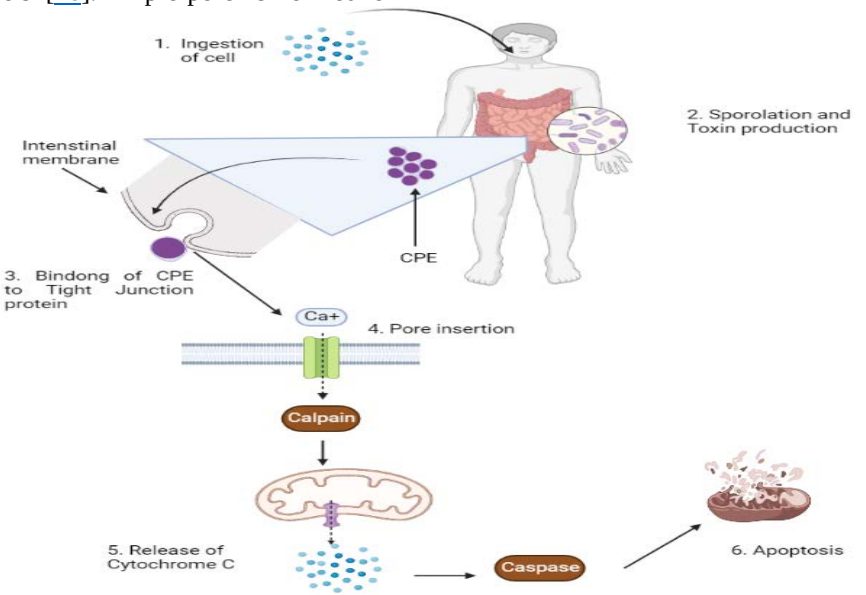

Figure 1. Schematic Diagram Showing Mechanism of Action of CPE on Cellular Functions [29]

The systemic diagram shows CPE binding to form a tight junction and pre-pore formation. The activation of calpain is followed by the release of cytochrome C from mitochondrial membrane. Final step ends with apoptosis.

6.1.2. Cell Death Mechanisms. Low CPE exposure causes a small amount of tiny pores to develop and a minor calcium ingress, which produces low level of calpain stimulation and apoptotic mechanism, shown by the removal of cytochrome and the engagement of caspase-3 [30]. Meanwhile, higher CPE dosages lead to the creation of many holes, a huge calcium inflow, substantial calpain expression, little caspase-3 stimulation, and morphological cellular alterations indicative of necrosis [30].

6.2. *C. perfringens* Alpha Toxin (CPA)

Alpha toxin is the main reason behind the disorganization of cell membrane. It contains phospholipase sphingomyelinase that causes hydrolyzation. This toxin has a

critical role in gangrene and acts as hemolytic. All *C. perfringens* isolates include the gene for CPA, which is located in a constant area of the bacterial genome [1]. An amino acid zinc metalloenzyme called CPA attaches to the target cellular membranes when Ca ions are found [31]. The latter alone has immunoprotective properties [1].

Inside the plasma membrane, the relative amount of sphingomyelin (SM) to phosphatidylcholine (PC), as well as the levels of local toxins, affect the action of CPA, which is incredibly complicated and different in different cell types. Due to such causes, CPA activity affects a variety of routes (Figure 2). CPA dissolves PC and SM in the plasma membrane to yield ceramide (CER) and diacylglycerol (DAG) [32]. The central looping domain of the toxin's ganglioside-binding region encourages tropomyosin receptor kinase A (TrkA) to engage with and connect to the cytoplasmic membrane [33], which results in membrane disruption. Although, membrane disruption is not the only way that CPA acts. The TrkA is encouraged to interact with and attach to the cellular membrane by the toxin's ganglioside-binding region in the central loop domain [33], which activates the MEK/ERK pathway. These actions could prevent CPA's ability to break cell membranes, thus shielding cells from cellular harm. The elimination of sialic acids by *C. perfringens* sialidases improves cell susceptibility to CPA *in vivo* and *in vitro* [34], suggesting a possible synergistic effect between sialidases and CPA. The sialic acids are crucial for gangliosides formation [35].

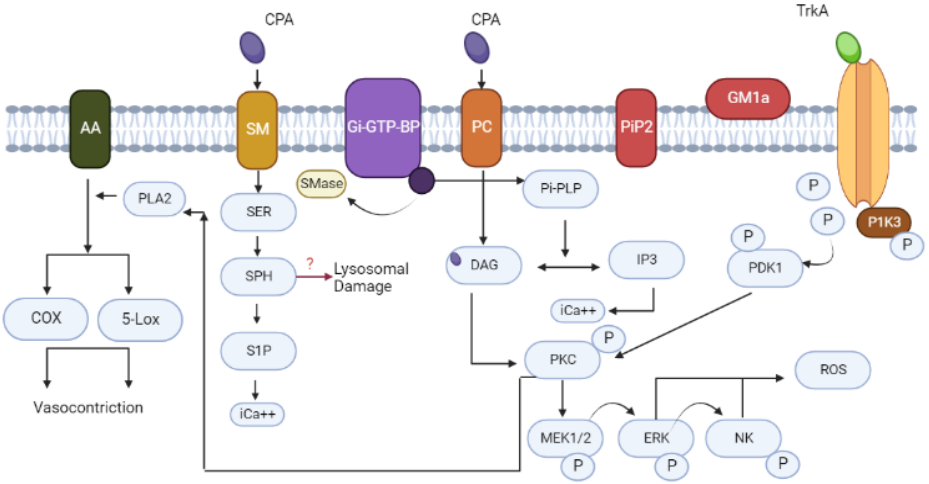

Figure 2. Schematic Diagram Showing the Mechanism of Action of CPA on Cellular Functions [29]

Sphingomyelin (SM) and Phosphatidylcholine (PC) are located in the cellular membranes of cells immediately hydrolyzed by CPA. Additionally, CPA may trigger the plasma membrane's Gi-type GTP-igatingl protein (Gi-GTP-BP). This, in turn, activates the body's own sphingomyelinases (SMase) and phospholipases (PI-PLC). Diacylglycerol (DAG) and inositol trisphosphate (IP3) are produced as a response to phospholipase operation; the

latter raises and mobilizes intracytoplasmic calcium ions (iCa2+). Sphingosine (SPH), ceramide (CER), and sphingosine-1-phosphate (S1P) are produced as a result of sphingomyelinase activity. Additionally, CPA's association with both the TrkA receptors causes the activation of PKC and PDK1. This activates the ERK/MEK signaling cascade as well as NF-B, thus activating the generation of reactive oxygen species (ROS) and IL-8.

6.2.1 Cell Death Mechanisms. Lytic doses of CPA can cause substantial cellular membrane breakdown and also the release of hydrolytic enzymes (LDH), which is indicative of necrotic activity [36]. Reactive oxygen species and MEK and ERK pathway initiation are linked to sub-lytic quantities of CPA [23] that, at some values, can induce oxidative stress in molecules and trigger innate pathways of apoptotic cell death [36]. Whenever CPA enters into a new cell through caveolae that comprises cholesterol, it stimulates signal transduction pathways along its action routes, while ROS generation can also result in lysosomal destruction [37]. High (iCa2+) or intracytoplasmic Ca2+ concentrations are involved frequently in pre-lethal apoptotic and necrotic processes [38]. From SPH and inositol trisphosphate (PIP2), CPA causes the synthesis of sphingosine-1-phosphate (S1P) and IP3, both of which are linked to the mobilization and elevation of iCa2+ [39].

6.3 *C. perfringens* Beta Toxin (CPB)

C. perfringens types B and C generate CPB, which causes illness in a number of animal species as well as human beings. They also cause enterotoxaemia and necrotic enteritis in numerous animal species. Such as in sheep, type B strains have been linked to lethal hemorrhagic dysentery [40]. The gastrointestinal pathogenesis of type C isolates gastrointestinal loops in goat and bunny. These are the natural hosts for *C. perfringens* type C illness, which could only be reproduced by CPB, according to a study which employed isogenic-null variants [41]. Additionally, CPB was demonstrated to be the cause of death in a mice enterotoxemic model [42].

6.3.1 General Pathway of Action. Bleeding and demolition of the small and (infrequently) large intestine's mucosa expresses the pathophysiology of uncontrolled illness linked to CPB. Harm fetched on by CPB starts in intestinal epithelium. It can blowout to the whole tract. Intestinal illness linked to CPB is characterized by fibrin thrombi that blocks the lamina propria's external microcirculation [2]. The wild-type C. perfringens type C strain CN-3685 caused destruction of the villi tip when thrombosis became evident in the rabbit intestinal loop model, indicating early intestinal mucosa damage [41].

Additional CPB immunohistochemistry research on pig jejunal micropropagation revealed that the toxin did not attach to epithelial cells. Further, the existence of a natural epithelial sheet prevented the identification of CPB in distal intestinal regions of the gut [43]. Yet, it was shown that CPB was not accountable for the reported cytopathic impact, since the culture supernatant of 2 *C. perfringens* type C strains caused IPEC-J2 cellular injury, which was not reversed by anti-CPB monoclonal antibody [43]. These membrane holes enable K+ escape and Na+, Ca2+, and Cl entrance into the cytoplasm, which results in cell edema [44]. Nec-1 inhibits autophagy, which is induced by an increment in iCa2+. Calpain stimulation and modest amounts of

caspase-3 triggering also happens, indicating that apoptotic activity is not a major cause of cell damage.

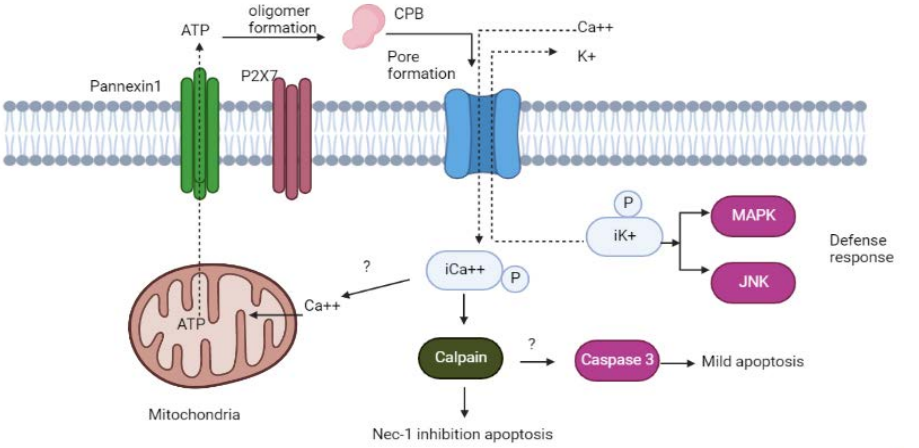

Figure 3. Schematic Diagram Showing the Mechanism of Action of CPB on Cellular Functions [29]

Targeted cells produce a quick surge of ATP upon initial CPB attachment to their ATP-gated P2X7 channel, which is one of their possible receptors. When pores develop, Ca2+ inflow occurs, while intracytoplasmic K+ (iK+) is lost. Nec-1 inhibits necrotic cell death, which is induced by an elevation in iCa2+. Calpain stimulation and modest amounts of caspase-3 initiation also occur, indicating that apoptosis is not the major cause of cell death.

6.3.2 Pathways of Cell Death. LDH secretion with propidium iodide (PI) ingestion are two examples of cellular activities that r-CPB quickly causes in porcine endothelial cells in vitro, according to previous investigations [45]. Both necrostatin-1 and calpain inhibitors prevent the two processes, indicating that CPB-prompted necrotic cell damage was not a gradual process; rather, it followed a predetermined metabolic route. It is still important to note because RIP-1, the necrostain substrate, can also play a role in cell killing [46].

6.4. *C. perfringens* Epsilon Toxin (ETX)

Considering its structural analogy to aerolysin generated by *Aeromonas* sp., this contaminant is categorized as the heptamer of the aerolysin class [47]. The primary pathogenicity component for all abnormalities and associated symptoms with *C. perfringens* type D enterotoxaemia has been identified as ETX by studies utilizing isogenic *C. perfringens* ETX-null variants in sheep, lambs, and mouse models [4].

6.4.1 General Mechanism of Action. Utilizing cell lines with renal origin from several organisms including rodents, dogs, and humans, wherein spontaneous occurrences of type D enterotoxaemia have yet to be documented, the adsorption and lethal effects of ETX were thoroughly

examined. It was found that renal cell lines from animals, such as sheep and cows, that are typically exposed to ETX are resilient [48].

According to a recent investigation, ETX stimulates neutral sphingomyelinase which further makes ceramide in the cell membrane to aid in oligomer production [49]. For example, in ACHN cells, the inhibition of neutral sphingomyelinase prevents oligomer synthesis and ETX-triggered cell death (Figure 4) [50]. Numerous cells that ETX is known to attack, such as intestinal epithelium, kidney cells, and neural stem cells, produce MAL. MAL is not expressed in synapses which have a rumored but unconfirmed sensitivity to ETX [51]. MAL might function as a particular ETX receptor, a protein responsible for the formation of the multiprotein complex necessary for ETX association with the plasma membrane, or perhaps it might also be involved in processes that are not related to pore creation, according to certain theories [50, 52].

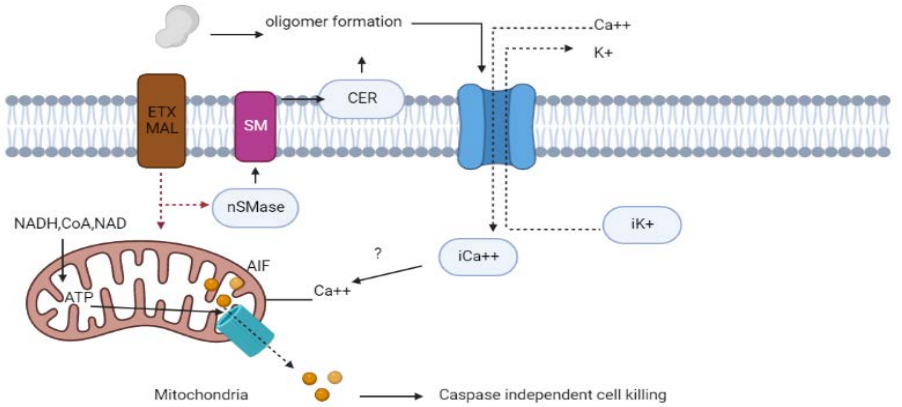

Figure 4. Schematic Diagram Showing the Mechanism of Action of ETX on Cellular Functions [29]

Attachment to its ligand (ETX-r), such as myelin and lymphocyte (MAL) proteins, is a necessary step in the activity of ETX (PDB ID: 1UYJ). A heptameric pore created by oligomerization causes a quick loss of iK+, entrance of Cl+ with Na+ (not illustrated), as well as a subsequent rise in iCa2. It would make it easier for the apoptosis-inducing factor (AIF), a factor that triggers cell death irrespective of caspase, to go from the mitochondrion to the nuclei.

6.4.2 Cell Death Mechanisms. The molecular pathways connected to ETX activity and the processes of cell death have not been thoroughly described yet. Infected cells develop pores, which cause an immediate depriving of internal potassium ions, an entrance of chlorine and sodium ions, and a subsequent rise in iCa2+ [53]. It has not been shown yet if the disparity of intracellular electrolytes is responsible for triggering particular downstream signaling processes that result in cell death. Specifically, coenzyme A along with nicotinamide adenine dinucleotide (NADH and NAD+) are lost in ETX-contrived cells, which contributes towards depleting the potential of mitochondrial membrane, as

well as the activation of the transition pore permeability. A powerful caspase-independent cell death protein called apoptosis-inducing factor (AIF) is also transferred from the mitochondria to the nucleus (Figure 4) [54].

6.5 *C. perfringens* Iota Toxin (ITX)

Type E strains of *C. perfringens* generate ITX. Intestinal illness has been linked to this toxinotype in a wide range of animal species. Yet, the identification of this toxin in the majority of these cases relied solely on the discovery of *C. perfringens* type E in the gastro-intestinal tract of animals suffering from bleeding inflammatory intestine. It is still uncertain how ITX specifically contributes to illness [55]. Following the discovery of ITX in the gut of sick animals, *C. perfringens* type E was once presumed to be the cause of enterotoxaemia in bunnies.

6.5.1 General Mechanism of Action. According to reports, Ia possesses a cellular receptor known as the lipolysis-stimulated lipoprotein receptor (LSR) which facilitates the toxin's entry into target cells. Additionally, it was demonstrated that endocytosis linked to the cell-surface antigen CD44 can occur when ITX enters host cells. The enzyme Ia constituent is released in an ineffective state that requires the elimination of 9 to 11 N-terminal repeats by proteolysis [56]. The actin Arg at position 177 is covalently attached to an ADP-ribose molecule via the Ia C-domain to provide the toxin's ADP-ribosylation action [57]. Increased porosity of intestinal cell monolayers in culture is brought on by changes in cell shape [58].

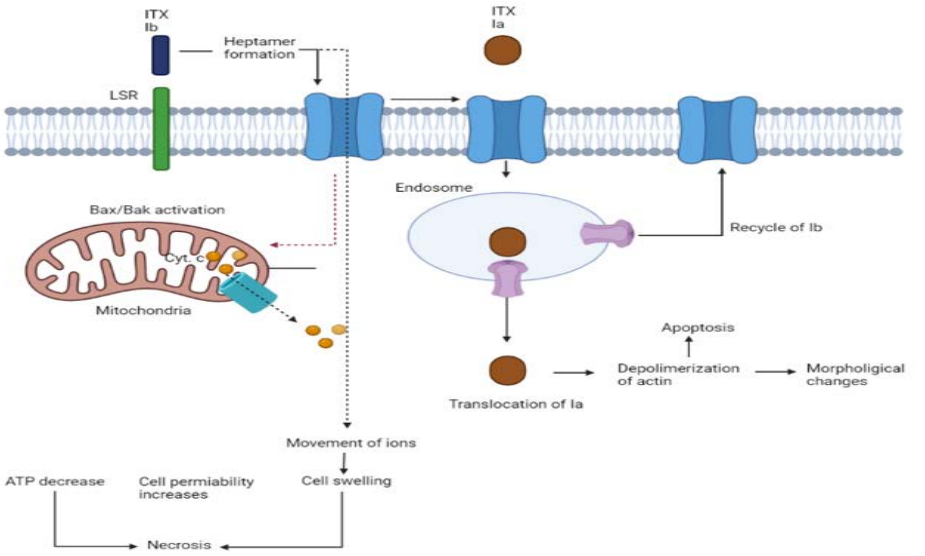

Figure 5. Schematic Diagram Showing the Mechanism of Action of ITX on Cellular Functions [29]

An enzymatic constituent (Ia) and a bonding element make up the combinatorial toxin known as ITX (Ib). It has been discovered that Ib binds to

lipolysis-induced lipoprotein receptors (LSR). Cytotoxic activity comprises necrotic characteristics, such as swelling, enhanced permeability, and reduced ATP in the cells. Additionally, cytochrome C is released from mitochondrial organelle as a result of the stimulation of Bak and Bax.

6.5.2 Cell Death Mechanisms. Different human cell lines, namely A431 and A549, show that Ib may elicit cytotoxic activity on its own [59]. These cytotoxic consequences include pronounced cell enlargement, mitochondrial failure, ATP shortage, and accelerated IP consumption — all indicators of necrosis. The pro-apoptotic components Bak and Bax were activated and cytochrome C is released, although caspase-3 is not activated. Moreover, incubating cells with the pan-caspase inhibitor ZVADFMK did not shield them from the Ib-induced decrease of survival. Furthermore, the internalization of Ib is necessary for cell vitality, pointing to an endocytic function in preventing Ib-related pore development in organisms.

7. HOST SPECIFICITY

As described earlier, toxins are of different types and found at distinct places. Type A is mostly detected in animal intestines and also in the environment. Type D is commonly detected in sheep. Type C is observed in pigs, while type E is observed in calves [60].

8. CLASSICAL AND MOLECULAR IDENTIFICATION OF *C. PERFRINGENS*

8.1 Molecular Genetics

Plasmid coding was reported at the molecular level in this pathogen's bacteriocin production. Its transferring activity was elucidated and cell to cell connection was revealed to be significant. It also plays a crucial function in caseinase activity. Experiments indicated that around 6% of fecal samples contain the enterotoxin gene. These samples came from animals [61].

9. ISOLATION AND IDENTIFICATION

9.1 Direct Plating Technique

Plating media was proposed for the identification of this pathogen. Black colonies were produced on medium. The criteria involved the existence and isolation of microorganisms having the same serotype as of the patients. However, some strains remained unidentified, which was considered as a limitation [9].

9.2 ELISA Technique for Enterotoxin Detection in Feces

The technique developed for the identification of enterotoxin is ELISA. It is used widely but latex agglutination kit is considered more convenient. Direct analysis of feces in patients showed the presence of enterotoxin. This toxin is also observed in the fecal sample of healthy persons but in a low quantity [61].

9.3 PCR Assay for Isolation

Enterotoxin is limited to a small ratio in isolates causing foodborne outbreaks through spores. So, for the identification of suspect isolates, polymerase chain reaction (PCR) assay was used in this study. This technique amplifies CPE from the cells that are lysed and gives information about the presence of organisms. It can also tell the location of CPE. As explained earlier, it is present on chromosomes or plasmid [62].

9.4 Confirmatory Tests

Confirmatory tests are performed at the end and they may detect the presence of sulfite reducing bacteria. Media used in this

case were motility nitrate and lactose gelatin. Both of them were used along with TSC agar recommended by the Association of Official Analytical Chemists. Some kits for recognition and identification are also present commercially for transposons of *C. perfringens* [63].

10. IMPORTANCE OF *C. PERFRINGENS*

10.1 Medical Applications of Enterotoxin

Enterotoxins are used for cancer treatment. Enterotoxin binding domain binds to claudins. Claudins are membrane proteins. Enterotoxin can be used as an anti-cancerous agent. Tumor necrosis in the case of pancreatic cancer has been observed. Recent studies on immune responses and toxicity to enterotoxin are ongoing [64].

C-CPE can be used to increase paracellular permeability of chemotherapeutic agent. It also has other medical applications, such as vaccination and drug absorption capability [65], as shown in Figure 6.

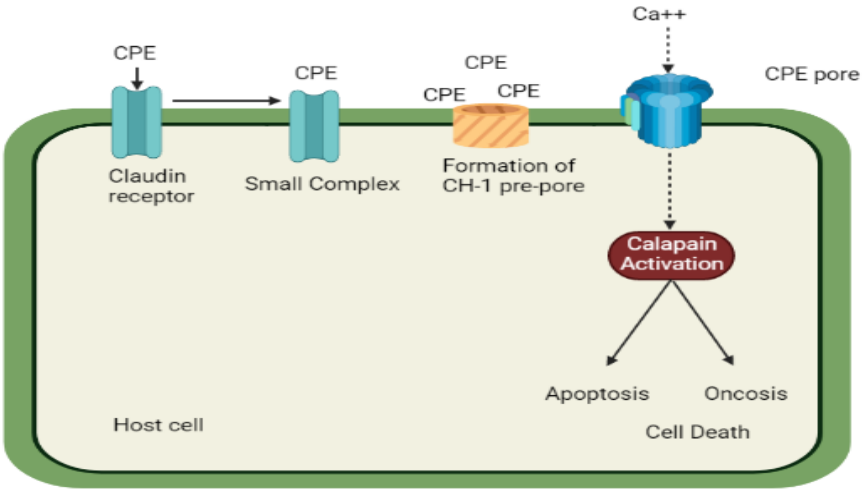

Figure 6. Schematic Representation of Cytopathic Effect Caused by CPE [29]

CPE initially forms a 90 kDa tiny compound by binding to claudin sites. The CH-1 pre-pore presumably forms on the surface of the membrane by the oligomerization of six tiny complexes. The active pore is created when the pre-pore is inserted into the membrane. This causes ca to enter the cells, activating calpain. A high CPE dosage produces significant ca entry into cells, which strongly activates calpain and causes cell damage through oncosis. Lower CPE dosages result in a weaker calpain stimulation and a greater constrained calcium inflow, which leads to the traditional caspase 3-induced apoptosis that kills such cells.

11. CONCLUSION

C. perfringens can be a major cause of economic loss by adversely affecting livestock. It has diversified toxinotypes that cause illness in human beings. It also causes mild food poisoning and remains resistant to high temperature. This capability makes it a good indicator in environment and water. Enterotoxins

produced by this pathogen have been observed in many fecal samples taken from ill persons. Significant advances have been made over the last several years in comprehending the intricate intracellular processes that contribute to this result. However, there continues to be a lot of unknowns in this field. Further analyzing the intricate relationship of *C. perfringens* toxins with their target cells may provide new pharmaceutical approaches regarding both human beings and livestock.

11.1. Future Perspectives

Knowledge about the factors that are superintended for host specificity is very limited. Two new toxins have been proposed in this study but there are chances of new toxinotypes of *C. perfringens* being discovered. Whether the genes present on extrachromosomal elements interact with toxin encoding gene or not still remains unclear. Type B causes dysentery in sheep but it is possible that it may cause it in other species as well. However, there is a need for further research at molecular level. There is still no knowledge of strain E virulence in livestock, although it is detected in rabbits and lambs. This strain releases an important toxin, which should be investigated in future research, as it is one of two medically important toxins (Tpel).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVALIABILITY STATEMENT

There is no data associated with this study.

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