



# To Study *In Vitro* Anti-proliferative and Pro-apoptotic properties of *Salmonella typhi* in Human Pancreatic Cancer Cell Line

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## Abstract

**Background:** Pancreatic cancer is one of the most common types of cancer that affects the gastrointestinal tract and the induction of apoptosis in cancer cells is generally one of the suitable methods for treating cancer. *Salmonella typhi* induces apoptosis by activating the caspase pathway.

**Methods:** This experimental study was conducted on a standard strain and the bacteria were cultured on an SS-Agar medium. Then, sonication was used to isolate bacterial proteins and ZellBio was utilized to determine the concentration of such proteins. In addition, 1 million cells per well were cultivated in the cell culture plate in order to treat the cell line with bacteria and to examine the expression of the *Bax* and *Bcl-2* genes. Then, RNA was extracted and cDNA was synthesized after 24 hours of incubation. The *Bax* and *Bcl-2* genes were expressed by Real-time polymerase chain reaction (RT-PCR).

**Results:** The findings confirmed the role of *Salmonella typhi* proteins in inducing apoptosis in a pancreatic cancer cell. In other words, the concentration of 10 mg *Salmonella typhi* proteins increased the expression of the *Bax* gene while reducing the expression of the *Bcl-2* gene.

**Conclusions:** Overall, bacterial treatments have unique mechanisms for treating cancer. For example, bacteria can specifically target the tumors, actively penetrate into the tissue, and potentially form a controlled form of toxicity. Accordingly, further studies are recommended to investigate the role of bacteria, especially *Salmonella* in the treatment of cancer cells.

**Keywords:** *Salmonella typhi*, RT-PCR, Pancreatic cancer, Bacterial-induced Apoptosis, Cancer treatment



## Background

Cancer can be defined as uncontrolled cell division under the influence of genetic and/or epigenetic factors such as environment and lifestyle (1,2). Pancreas usually forms cellular mass when the pancreas defected cells continue to divide and scape the programmed cell death. This recently formed tumor can become metastasis as well (3,4). In addition, pancreatic cancer prevalence is higher in the elderly and around half of these people are above 75 years of age. However, this type of cancer is seldom observed in people under 40. Gender specificity is off the table in this cancer and both genders have an equal chance of catching pancreas cancer. There is no obvious sign and the symptoms are tumorigenesis at the early stages of this cancer, therefore, it is one of the main reasons which makes the early diagnostics almost impossible (4). Data analysis suggests that most of the cancers happen to be the exocrine pancreatic part and their most common symptoms are back pain, abdominal cramps, jaundice, and weight loss (5). By considering the aggressive nature of cancer yet

complex mechanisms, conventional treatment options are surgery, as well as chemo and radiotherapy, but they are short-time alternatives and mostly less effective or even ineffective. Several drawbacks of the above-mentioned methods include the lack of specificity, the presence of numerous side effects, and the chances of metastasis. Thus, finding an effective method enhances the specificity and minimize the side effects for greater results, suppressing the uncontrolled growth (6,7).

Since the early 1990s, proteins, bacterial toxins, and the like came to light as a potential treatment for cancer (8). So far, researchers have identified more than 100 genes which play an undeniable role as the protector or guardian of the cell and its longevity. Apoptosis is a mechanism for excreting defected and unhealthy cells in eukaryotes including the human. Further, the suppression of apoptosis is a fundamental factor in human malignancies. Cancer cells can escape apoptosis which will turn the cell into the fetal accumulated mass of cells which are known as the tumor. Two large families of genes are involved in

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the apoptosis pathway, namely, *Bcl-2* and caspases. *Bcl-2* protein plays a dual role either pro or anti-apoptosis. Similarly, *Mcl-1*, *Bcl-2*, and *Bcl-xL* association has an anti-apoptosis impact while proteins like Bax, Bak, Bad, and Bim are the key players in the pro-apoptosis side (9,10). The induction of apoptosis is one of the most well-known options to consider in cancer treatment since it has the minimum risk including less side effects and a plus point like higher specificity. Researchers are in a race against time for finding the best compound to nail the goal. These compounds can be either the extracts of medicinal plants or inorganic compounds. As previously mentioned, the blooming idea of bacterial-mediated apoptosis has come under the spotlight since almost three decades ago and many advances have so far been made in this regard. *Salmonella* is one of those successful attempts which can be a prominent choice in cancer treatment, along with its pathogenicity (11). Furthermore, *Salmonella* belongs to Enterobacteriaceae but it is simultaneously considered as the gut bacterium of humans and animals (12). *Salmonella typhi* and *Salmonella Paratyphi* are the main pathogens of typhoid fever (13). The gram-negative bacilli are non-spore-forming, which are encapsulated and motile. *Salmonella* is regarded as a facultative anaerobe as well (14). Previous studies have rarely investigated bacterial-mediated apoptosis induction on pancreatic cell lines compared to the other cancer cell lines. Pancreatic cancer by 338 000 new cases per year is the 12th pervasive type of cancer. Accordingly, for this study, the pancreatic cancer cell line was chosen to evaluate its effectiveness, measure the expression ratio of Bax/Bcl2 by the latest and sensitive methods (e.g., real-time polymerase chain reaction and the like), and thus assess the outcome and elucidate how this gram-negative bacterium can alter and affect the apoptosis pathway.

**Materials and Methods****Sample Procurement**

In this experimental study, the Aspc-1 cell line and ATCC certified *Salmonella typhi* bacteria were purchased from the Iran Biological Resource Center and the Pasteur Institute of Iran. Then, a biochemical assay was performed to assess the purity of the obtained species.

**Obtaining Bacterial Protein**

To this end, 2  $\mu$ L of sterile PBS buffer was added to a 5 mL bacterial pellet and sonicate at 250 W intensity for 5 minutes with 30-second intervals. Next, the mixture was centrifuged at 8°C at 9000 rpm for 10 minutes. Then, the supernatant was harvested by a sterile syringe, followed by filtering and transferring it into a micro tube and storing it at -20°C. Eventually, protein concentration was analyzed by Bradford assay (ZellBio).

**MTT Assay**

The Aspc-1 cell line was cultured overnight in RPMI (Sigma-Aldrich, Germany) supplemented with 10% FBS (Gibco, Grand Island, NY), 1% penicillin (100 U/mL), and streptomycin (100 g/mL, Biosera, UK) and then were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> and 100% humidity. The medium was replaced with the fresh one in every 48 hours before any treatment aiming at covering the surface of the flask for 80% by the culture cells. Next, Aspc-1 cells were seeded on six-well plates at a density of 1.0×10<sup>6</sup> cells/well leave first well for untreated (control) and the remaining plates/cells were filled up with different concentrations of bacterial extract (i.e., 10 and 100  $\mu$ g, along with 1, 5, and 10 mg). After 24 hours of incubation, the cells were washed 3 times with PBS and all media were replaced by fresh media, followed by the addition of MTT reagent to each well. The cells were further cultured for 4 hours and then all of the media was replaced with dimethyl sulfoxide. After 30 minutes of incubation, the six-well plates were shaken strongly and the absorbance was measured in an Elisa Reader (SpectraMax M2e, Molecular Devices) at 570 nm.

**Real-Time Polymerase Chain Reaction (RT-PCR)**

The qRT-PCR was performed on Aps1 cells for 24 hours in order to determine the effect of the extract of *Salmonella typhi* on the mRNA expression of *Bcl-2* and Bax. After treatment, the contents of each well were transferred to a micro tube and stored at -20°C. Later, 200  $\mu$ L of chloroform was added to each 1.5 mL micro tube and it was centrifuged at 12000 rpm for 5 minutes at 8°C, followed by obtaining three phases of phenol, protein, and RNA. The supernatant was relocated to a new micro tube and top up with isopropanol and kept at -20°C for 12 hours. Next, it was centrifuged at 12000 rpm for 15 minutes at 8°C after 12 hours. In addition, the supernatant was discarded and the remaining alcohol was let to evaporate and air dry. Finally, 30  $\mu$ L DEPC-treated water was added to the micro tube. The agarose gel electrophoresis was performed to confirm the RNA extraction as well. Further, the random hexamer primer kit (Thermosience, Lithuania, EU) was utilized as the protocol of per kit for DNA synthesis. Similarly, one-step RT-PCR was performed on by real-time PCR (Applied biosystem-USA) after the extraction of total RNA, and the reconfirmation of gene expression with RT-PCR. RT-PCR evaluation for all genes is listed in Table 1. For target sequence amplifications, 100 ng (5  $\mu$ L) of RNA was used per 2  $\mu$ L reaction volume as well. For each sample, the reference gene ( $\beta$ -actin) and the target genes were amplified in the same run, followed by obtaining standard curves using the logarithmic dilution series of total RNA. Eventually, PCR products were interpreted and analyzed by the REST software and the results of less than < 0.05 were considered as desired outcomes.

Table 1. Primer Sequenced Used in Real-time-PCR

	Gene	Primer Sequence	Melting Temperature (Tm)	PCR Product Size (bp)
<i>Bax</i>	Forward	5'- TGGGCTGGACATTGGACTTCC -3'	61.77	173
	Reverse	5'- GCCTCAGCCCATCTTCTTCC -3'	60.39	
<i>Bcl-2</i>	Forward	5'- ACTTGACAGAGGATCATGCTG-3'	62.31	169
	Reverse	5'- ACTTGATTCTGGTGTTCCTCC -3'	62.96	
<i>Beta-actin</i>	Forward	5'-TCCTCCTGAGCGCAAGTAC-3'	62.10	155
	Reverse	5'-CCTGCTTGCTGATCCACATCT-3'	60.34	

Abbreviation: PCR, polymerase chain reaction.

## Results

### Apoptosis Analysis

The existence frequency of Aps-1 cells was calculated by incubating the cells with different concentrations of bacteria protein extract (24 hours). The protein extract could prevent the proliferation of Aps-1 cells at concentrations of 5 and 10 mg/mL. Figure 1 displays dose-response behavior against the cells.

### mRNA Expression of Selected Apoptosis-Related Gene Transcripts

The formation of 16 sRNA and 28 sRNA bands confirmed that RNA extraction and electrophoresis were performed correctly (Figure 2).

Based on data analysis and interpretation, the expression ratio of genes in the treated samples by 10 and 100 µgm showed no significant changes compared to the control group ( $P>0.05$ ). On the other hand, samples treated by higher doses (e.g., 5 and 10 mg) of *Salmonella typhi* protein demonstrated up-regulation in *Bax* gene ( $P<0.05$ ). The gene expression amount at 5 mg was 1.905 whereas the highest expression changes was related to 10 mg concentration of *Salmonella typhi* bacterial protein which was equal to 11.551. The gradual expression of *Bcl-2* gene at 10 µg treated samples represented negligible changes in comparison to the  $\beta$ -actin gene expression of the control sample. Furthermore, no noticeable changes were found ( $P>0.05$ ) at 100 µg and 1 mg of *Salmonella typhi* concentrations. The highest amount of decline in the down-regulation of the *Bcl-2* anti-apoptosis gene belonged to 5 mg concentration of bacterial extract by the mean value of 0.352.

As illustrated in Figure 3, the up-regulation of *Bax* gene was more than the other genes, which confirmed the positive apoptosis induction and incline in the rate of the programmed cell death. Moreover, *Bcl-2* -5 was another gene with significant changes is. The down-regulation of this gene increased the apoptosis by considering the anti-apoptotic properties of the *Bcl-2* gene. However, there were no such remarkable changes in the expression of *Bax1*, *Bcl-2* -1, and *Bcl-2* -2. Additionally, no suggestive changes were observed in the other involved genes compared to

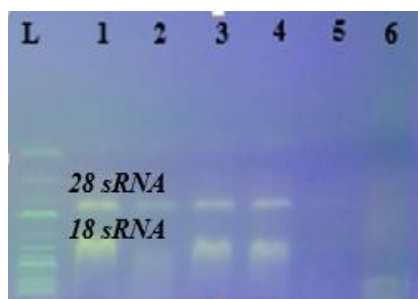
the control ones. Therefore, 10 mg concentration of the bacterial extract used to treat the pancreatic cancer cell line enhanced the apoptosis rate and thus suppressed the growth of cancer cells. furthermore, up-regulation and down-regulation of *Bax* and *Bcl2* genes shown in figure 4 and 5 respectively.

## Discussion

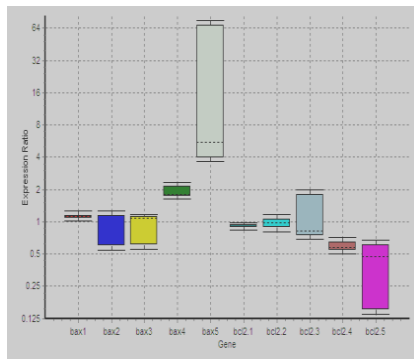
The prevalence of pancreatic cancer has demonstrated an increase in recent years and, according to the World Cancer Research Fund International, its mortality ranks 12 after lung, breast, colorectal, prostate, stomach, liver, cervix, oesophagus, bladder, non-Hodgkin lymphoma, and leukaemia. Apoptosis follows unique yet complex mechanisms to nail the programmed cell death without inflammation, unlike necrosis. In addition, apoptosis is generally defined as the removal of unwanted or defected cells in eukaryotes or even some prokaryotes. Nowadays, scientists employ apoptosis inducer or occasionally apoptosis suppresser genes in in vitro studies in order to direct the immune system toward the intended pathway regarding establishing the balance between cellular and humoral immunity responses. Further investigation continues in vivo to expand the horizon and evaluate the chances for clinical trials (16). The results of the current study are in line with previous findings and confirm the positive role of *Salmonella typhi* bacterial extract in the induction of apoptosis. For example, Raymond et al. studied mitochondrial-mediated apoptosis which activates caspase pathways by a bacterium like *Salmonella typhi* and reported significantly decisive results (17,18). In addition, this bacterium is found to affect *Bax/Bcl-2* gene expression, directly or indirectly (19). Likewise, the effect of *Salmonella typhi* bacterial extract was undeniable on the expression of the above-mentioned genes in our study. Further, Chorobik et al, by analyzing all species of *Salmonella* in spite of their pathogenicity, as well as considering their role in triggering mitochondrial-dependant intrinsic pathway could nominate *Salmonella* as a potential treatment option in some cancers (20). Another study on the remedial properties of *Salmonella* in China demonstrated that this bacterium can activate exclusive mechanisms to achieve its apoptotic purposes



**Figure 1.** Pancreatic Cancer Cell Line Treated With Different Dosages of *Salmonella typhi* Bacterial Extract.

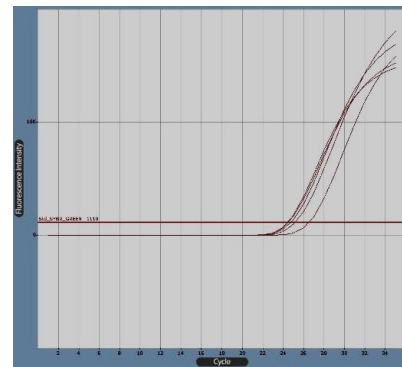


**Figure 2.** The Result of *Salmonella typhi* RNA Extract (R ladder 1 kb).

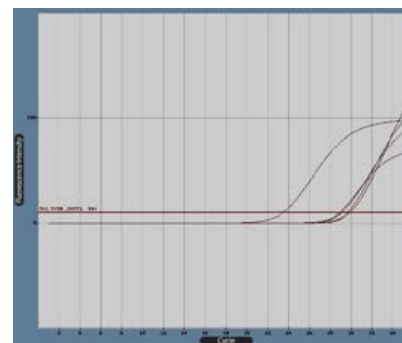


**Figure 3.** X Axis Showing Different Baxes Stands for 50% of the Median of the Observed Dots Partitioning Between Gene Expression Shadows Representing the Minimum and Maximum Rate.

(21). Likewise, our findings suggested that we can employ this property against cancer and rely on in therapy by suppressing the pathogenic factors, and subsequently, diminishing cytotoxicity. The results further proved that bacterial protein sparks off apoptosis pathways by affecting the expression of *Bax* and *Bcl-2* genes. The first trials have been performed on the tumor-fighting properties of *Salmonella* species since the early 1990s, and recently, few species of *Salmonella* like VNP20009, A1-R, and CRC2631 have received all the approvals for use in chemotherapy (22). While studying the embryonic fibroblast (inoculated to *Salmonella typhimurium*) of



**Figure 4.** *Bcl-2* Gene Boost Diagram in Real-Time Polymerase Chain Reaction by Cyber Green Fluorescence. Note. The diagram was produced by LightCycler software .



**Figure 5.** *Bax* Gene Boost Diagram in Real-Time Polymerase Chain Reaction by Cyber Green Fluorescence. Note. The diagram was created by LightCycler software.

the rat, Ruan et al found that TRAF6 (TNF receptor-associated factor) could increase the apoptosis rate by ROS accumulation, which gradually led to an elevation in *Bax/Bcl-2* ratio in the mitochondrial membrane and released cytochrome C to the cytoplasm (23). Furthermore, proteins such as *Bcl-2* play a crucial role in the permeability of mitochondrial outer membrane in the cells and can even modify the dynamics although its molecular mechanisms are still not fully known (24). *Bcl-2* protects the cell by suppressing the apoptosis pathways (25) while the *Bax* gene regulates the apoptosis (26). Our findings indicate that the bacterial protein of *Salmonella typhi* induces the apoptosis in the pancreatic cancer cell line. Based on the recent trials in Massachusetts in the United States, there is a possibility of employing a few types of bacteria together in cancer treatment (16). In the field of bacterial-mediated apoptosis, such bacteria can regulate a couple of unique pathways and speed up the apoptosis mechanism. The evidence further suggested that some of the infectious bacteria in certain measured doses can be used in the therapies by initiating apoptosis (18). Similarly, Camacho et al studied engineered *Salmonella* as the internal factory of a cell to excrete defected tumor cells. They reported that it is necessary to provisionally separate the produced proteins from the

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release proteins to effectively fight back against the tumor cells (27). Likewise, the extraction and separation of the bacterial protein of *Salmonella typhi* in the present study resulted in the induction of apoptosis in the pancreatic cancer cell line. Thus, our results confirm the key role of the bacterial protein of *Salmonella typhi* in the pancreatic cancer cell line. The above-mentioned extract has a remarkable influence on the Bax/Bcl-2 ratio and their expression. As mentioned earlier, *Bax* (pro-apoptotic gene) and *Bcl-2* (anti-apoptotic gene) genes up-regulate and down-regulated by 10 mg of sonicated bacterial protein, respectively. Apoptotic induction is considered a beneficial and useful method for removing the defected tumor and cancerous cells since the tumoral cells normally lack the apoptosis machinery or are somehow out of order. Thus, compounds like *Salmonella typhi* bacterial extract which can induce apoptosis, are extremely important since they act with higher specificity compared to radio or chemotherapy and have less known side effects. Bacterial products such as protein, enzyme, immunotoxins, and secondary metabolites are utilized since they exclusively target and remove cancerous cells either by their anti-proliferative characters, cell cycle arrest, or apoptosis induction. The recent investigation supports the idea of using the Bax/Bcl-2 measurement ratio as potential biomarkers in cancer prognosis or even prediction (28). The expression of pro and anti-apoptosis genes like *Bax* and *Bcl-2* in combination were found highly predictive and beneficial in colorectal cancer as compared to each gene expression separately (29,30).

**Conclusions**

Finally, we suggest further anti-tumoral compositions in this compound, therefore, future studies can be in vivo and probably human clinical trials. Based on the increasing rate of cancer worldwide and the burden of treatment expenses on individuals and governments, there is a growing need for such a cheap, handy, and conventional predictive method such as cancer biomarkers.

**Conflict of Interests**

The authors declare that there is no conflict of interest regarding the publication of this article.

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