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Cell death induction by Streptococcus pyogenes in four types of malignant cell lines

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Abstract

Background: The interest in using bacteria as anti- cancer therapeutic agents dates back to the end of the19th century. Some bacteria like Salmonella and Listeria replicate effectively inside malignant cell lines and suppress their growth. The bacterium Streptococcus pyogenes has become medically famous as a flesh-eating pathogen since mid-1980s. It is the causative agent of a life threatening clinical condition called necrotizing fasciitis. S. pyogenes usually produces a range of lytic enzymes that promote bacterial pathogenesis. With these characters, could this bacteria. be employed as a curing agent for certain cancers? The aim of this study was to determine the influence of S. pyogenes on malignant cellular death (apoptosis or necrosis)- in an ex-vivo "experimental- interventional" study.

Methods: The cytotoxicity of fifteen internalized streptococcal strains(including 12 clinical isolates, 2 known M types [M1, M3] and standard strain), on four types of malignant cell lines- A549, BT-20, PC-3, L-929- were tested by Trypan blue exclusion, DNA fragmentation and WST-1 methods. The streptococcal protease, lipase, DNase and serum opacity factor (SOF) were tested concurrently. The standard strain of Streptococcus (Enterococcus) faecalis was employed as negative control. The results were analyzed by statistical Minitab software.

Results: The overall cytotoxicity rate of -internalized- S. pyogenes was 57% by trypan blue method and 50 % by DNA electrophoresis. False positive results occurred for the negative control in WST-1; therefore this test did not present reasonable results. The correlation between production of SOF, lipase, DNase and cytotoxicity of S. pyogenes was not significant ($p > 0.05$). However, 67% of the protease positive strains induced cellular death in at least one type of - malignant cell line ($p<0.05$).

Conclusion: Our findings indicated that, some non-invasive S. pyogenes that cause benign infection like pharyngitis can induce cell death in various cancerous cell lines. It seemed that among bacterial products, the proteolytic enzymes- linked to the streptococcal pyrogenic exotoxin B (spe-B)- were more related to bacterial invasion.

Keywords: Streptococcus pyogenes, cancer treatment, malignant cell, apoptosis.

Introduction

Because of some problems in about fifty percent of the current cancer treatments, the alternative methods are being actively considered [1]. The interest in using bacteria as an anti-cancer therapeutic agent dates back to the end of the 19th century [2,3]. It has been shown that some bacterial strains like: Salmonella choleraesuis, Vibrio cholera, Listeria monocytogenes, and Escherichia coli replicate effective-

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ly inside malignant tumors [3,4]. Recently it was found that the metabolites of some sea-water bacteria and gliding bacteria have strong activity against human breast adeno-carcinoma, colon cancer, cervical cancer and oral cancer cell lines. The 16S rDNA sequencing analysis of these bacteria are related to Bacillus-vallismortis and Cytophaga-Flavobacterium-Bacteriodes respectively [5, 6]. The exact mechanisms of tumor suppression have not been fully understood [3]. Among pathogenic bacteria, the Streptococcus pyogenes (group A streptococci-GAS), is responsible for a wide range of human diseases [6]. Since mid-1980s S. pyogenes has become medically famous as a flesh-eating pathogen [8]. As it causes a life threatening clinical condition called necrotizing fasciitis [8]. Viable S. pyogenes have been found inside the epithelial, endothelial, neutrophil and some other cell lines. However, this bacterium is known as an extra-cellular microbial agent [7]. Among its toxin-like products, the streptococcal pyrogenic exotoxin B (Spe B), potentially enhances tissue damage [10,11]. Essentially, Spe B is a cysteine protease with a considerable role in streptococcal pathogenesis [8]. Other extra- cellular products that may promote cell invasion are lipase, DNase, SOF,... [10,12, 13,14]. However, these enzymes are not unique for streptococcal strains [14,15]. This study was conducted to determine the anti-cancer potency of S. pyogenes in an ex-vivo "experimental- interventional" trial. The main purpose was to test the cytotoxicity of some - internalized-clinical isolates of S. pyogenes on some carcinoma cell lines. The enzymatic properties of isolates were also analyzed concurrently.

Methods

Twelve clinical isolates of S. pyogenes (10 isolates from pharyngitis cases, 1 from a synovial culture and finally 1 from a blood culture) and 2 known M type strains (M1 and M3) that were kindly provided by the Pasteur Institute(Tehran- Iran) as well as the standard strain(ATCC 8668) were examined. The enterococcus faecalis standard strain (NCTC 8213) was employed as the negative control in all steps.

Initially, the stationary phase of bacterial growth was determined by standard growth-curve analysis [12]. Accordingly, a fresh suspension (Mc Farland No. 0.5) of the over-night (~18 hours) culture- in the Todd-Hewitt broth (Himedia-india) was prepared. The serial dilution was then prepared and used for each cytotoxicity or enzymatic test.

The Staphylococcus aureus (ATCC 25923) and Staphylococcus epidermidis (PTCC 1435) were used as the positive or negative control in some bacterial enzyme tests.

Bacterial biochemical tests

i) Serum Opacity Factor(-SOF), The overnight culture of bacterial sample in the Todd- Hewith broth was centrifuged, and then 0.1 ml of filtrated supernatant (0.45 μm, Millipore-USA) was added to 1 ml of sterile horse serum. After incubation (37° C) for 16-18 hrs, the opacity density of the mixture was evaluated with naked eye [14]. The M3 type of S. pyogenes and the Staphylococcus epidermidis were included as the positive and negative controls respectively [16,17].

ii) Protease test, A few numbers of fresh bacterial colonies were inoculated onto Litmus milk agar (BBL- UK) and incubated (37° C) up to 10 days [16]. Positive result of casein (protein) hydrolysis was indicated by the formation of a clear zone around colonies. Staphylococcus aureus was included as the positive control [15,19].

iii) *Lipase test,* Bacterial strains were first plated onto Margarine containing (1%) Brucella agar (Difco-USA). After incubation (48 hrs at 37° C), the cultures were kept in sealed plates for 2 weeks at room temperature [20]. The positive result was assessed by formation of iridescence zone and/or white precipitation around colonies [20]. S. aureus was included as the

positive control [15].

iv) DNase test, The bacterial strains were inoculated in DNase test media (Merck - Germany). After incubation (37° C for 24- 48hrs), the hydrochloric acid -HCl (1%) was added to culture plates. Formation of a clear zone around bacterial colonies was indicative of the positive result [21]. The S. aureus was included as the positive control [15].

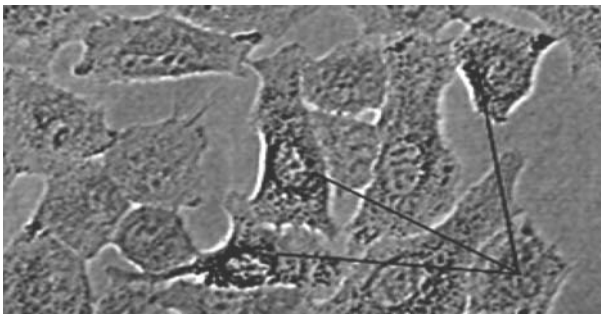

Fig1. Bacterial internalization into A 549 indicated by arrows

Strains	*Biochemical tests*			
	SOF	Zone of protease hydrolysis(mm)	Lipase	DNase
M_1 ⅄	-	0.6	+	+
M_3 ⅄	-	0.5	+	+
b300	-	0.6	+	+
b85	-	-	+	+
d72	+	0.8	+	+
d65	+	0.5	+	+
d21	-	0.5	-	+
p13	-	0.6	-	+
hos ⅄	+	-	+	+
n96	-	0.8	+	+
b242	-	-	+	+
b64	-	-	+	-
d25	-	-	+	+
1447★	-	0.6	+	-
ganji✷	+	-	+	+
E.faecalis✸	-	-	+	-

Note: The code of each isolate was chosen by using the first letter of the name of patient's physician + a chain number.
⅄: M types provided from Pasteur institute
⅄: isolated from blood
★ isolated from synovial fluid
✷: Standard strain of S. pyogenes (ATCC: 8668)
✸: Standard strain of E. faecalis (ATCC: 8213)

Table 1. The results of bacterial biochemical tests

Cell lines

To perform the research [19,22], four different types carcinoma cell lines including A549 (human respiratory epithelial cell), BT-20 (human breast epithelial cells), PC3 (human prostate epithelial cell) and L929 (mouse fibroblast cells) purchased from cell bank of Pasteur institute (Tehran-Iran) were examined. The cell culture used in Dulbeccos Modified Eagles Medium-DMEM (sigma-USA) was supplemented with 10% fetal bovine serum (FBS) in optimal condition (370 C, CO2 10% and humidity ~ 95%), and subcultures were done every 3 days by standard method [7,19, 23].

Bacterial internalization assay, the freshly prepared cell suspension was seeded in 24-well

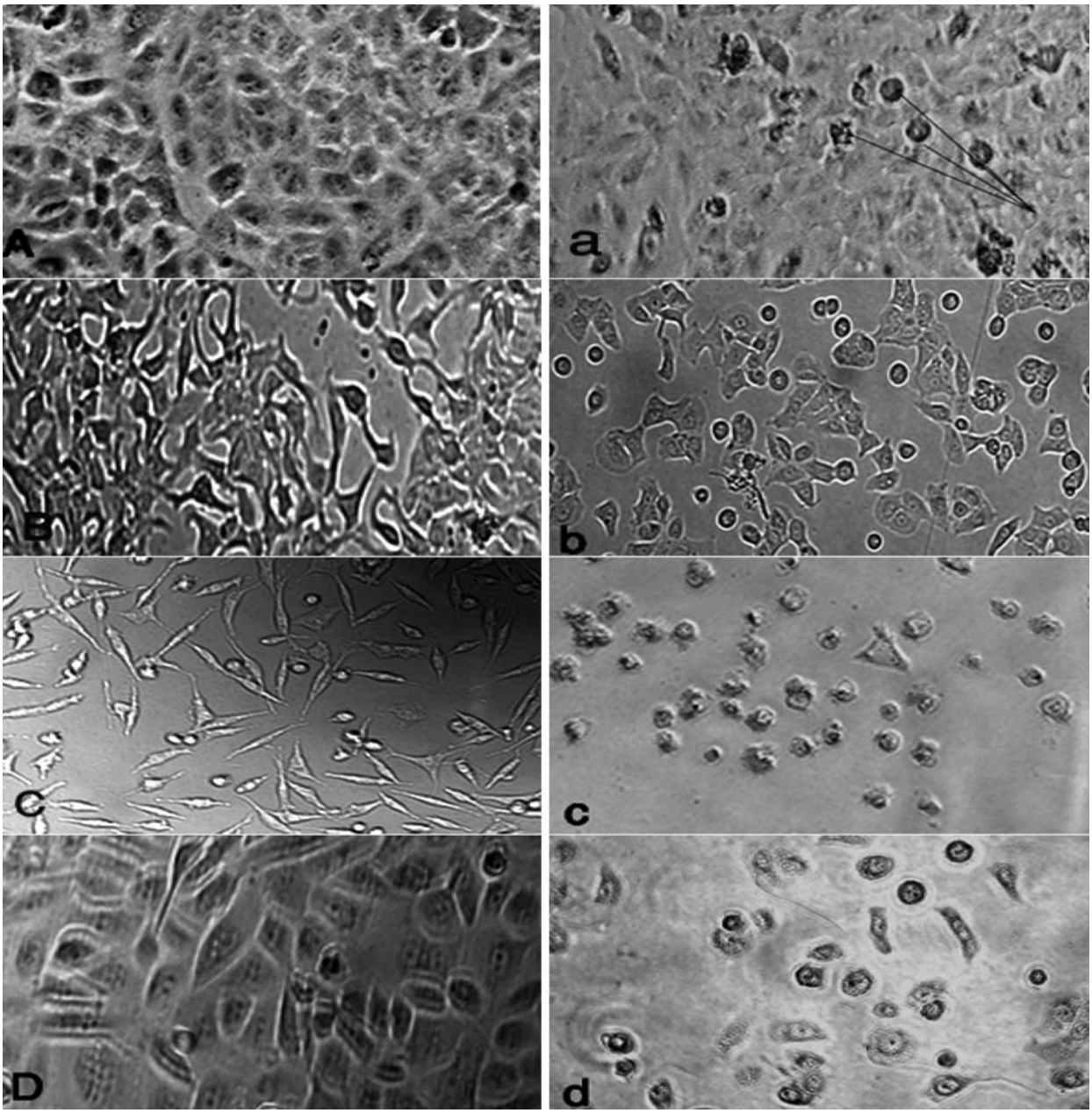

Fig. 2. Demonstration of A: un-infected, a: infected cell line (A549) B: un-infected, b: infected cell line (BT-20) C: un-infected, c: infected cell line (PC3) D: un- infected, d: infected cells line (L929).

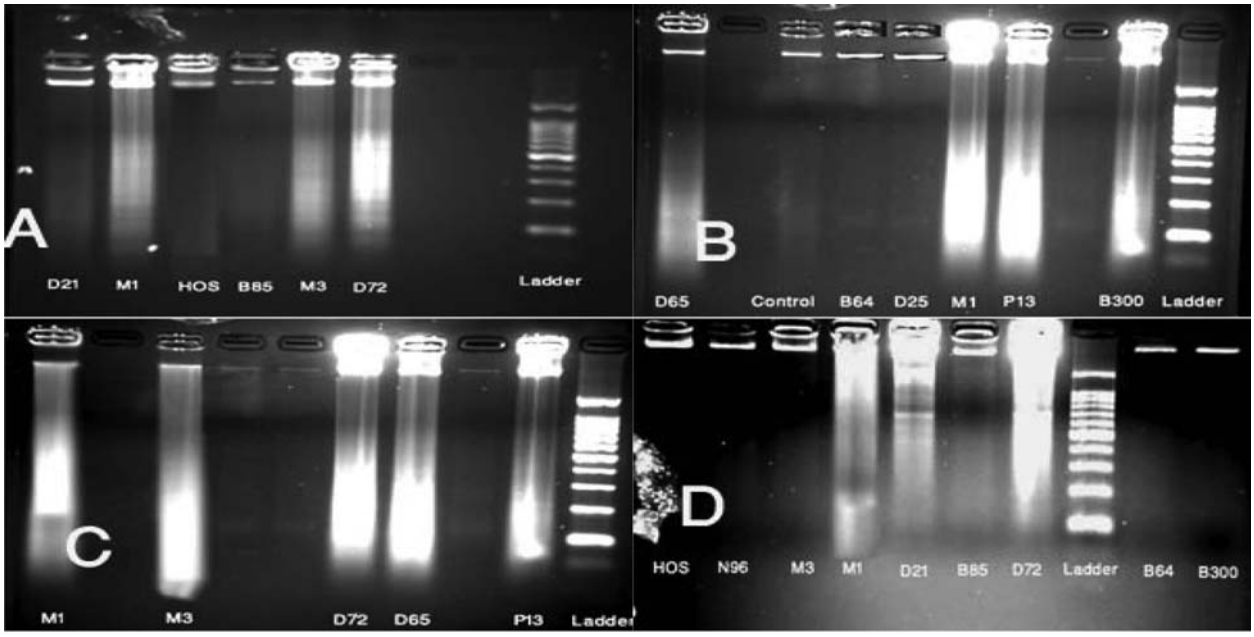

Fig. 3. A→D : formation of smear from degradation of DNA in apoptotic cells by 2% agarose gel electrophoresis.

tissue culture plates (~5× 10^4 /well) and incubated in optimal condition for 24hrs [19,22]. Then the prepared monolayers were infected with approximately 5× 107 bactria/well, to obtain a multiplicity of infection-m.i.o- of 1000 [19,22]. The mixture was incubated in DMEM + 20% FBS - without any antibiotics- for 2 hours (37° C, 5% CO2) [19, 24]. In order to eliminate unbounded bacteria, they were washed up 3 times and re- incubated in a penicillin containing fresh medium (3µg/ ml) for additional one hour [25, 26]. Finally, the infected monolayers were thoroughly rinsed with PBS and incubated in an off-antibiotic medium, for 24- 48 hrs [19,22]. The viability of the internalized bacteria was randomly monitored by the physical cell lysis method in ice- water and then the lysate inoculated on a blood agar plate [27].

Cytotoxicity Assays

i) Trypan blue exclusion stain, tripsizing the cell lines, the prepared cell suspension was mixed with an equal volume (1:1) of trypan blue- 0.4% solution. After 1-2 minutes, the number of total and stained cells were counted in the Neubauer chamber [22,28]. The calculated percentage of stained cells revealed the percentage of dead cells [28].

ii) Analysis of DNA fragmentation. For preparation of infected cells, the cell-lines were washed and trypsinized 24 hrs after incubation. DNA extraction was done by the manufacturer protocal (Qiagen- Germany). Electrophoresis of The extracted DNA was electrophoresed in the 2% agarose gel (Fermentas- Lithuania), and the gel product stained by ethidium bromide and visualized by UV in Gel-Doc [7,19, 22]. The non- infected cell line was treated in the same way as the negative control.

iii) WST-1 method. According to Patrick et al's proposed protocol [22], the prepared cell suspension was seeded in a 96-well micro-titer plate (~10^4/ well) and infected with S. pyogenes at m.i.o of 1000. The infectivity of the bacteria in cell line was continued as described above. After the planned time of incubation, the WST-1 dye (Roche- Germany) was added to each well, and the optical density (OD) of each well read with a kinetic micro-plate reader at a

Strains	Trypan blue staining % stained(dead) cells				Gel electrophoresis			
	A549	BT-20	L-929	PC-3	A549	BT-20	L-929	PC-3
M_1	40	40	40	50	+	+	+	+
M3	50	40	40	30	+	+	+	-
b300	70	20	30	20	+	-	-	-
b85	5	20	30	10	-	-	+	-
d72	40	60	60	40	+	+	+	+
d65	70	70	60	40	+	+	+	+
d21	10	60	20	20	-	-	-	+
p13	30	40	60	30	+	+	+	+
Hos	20	60	10	10	-	-	-	-
n96	20	5	20	20	-	-	-	-
b242	10	10	10	10	-	-	-	-
b64	20	20	10	10	-	-	-	-
d25	20	20	10	10	-	-	-	-
1447	50	60	30	30	-	-	-	-
Ganji	5	10	20	10	-	-	-	-
E.faecalis	20	20	20	30	-	-	-	-

Table 2. Comparison of the results of cytotoxicity of all 15 strains of S. pyogenes + standard strain of S. faecalis on 4 types of malignant cell lines by 2 methods: Trypan blue, and gel electrophoresis.

wavelength of 450 nm twice. First immediately after adding the dye then 1 hr after incubation (37°C) [22]. Triple cell cultures were tested for each cell line and repeated at least three times-for an individual isolate/strain- on different days.

iv)Assessment of the morphological changes. An inverted phase contrast microscope-equipped with a camera (Olympus IX71-Japan) was used to visualize any changes in the morphology and cytopathic effects (CPEs) of infected cell line. The assessment of CPE provided a useful way for following up of cell lines infectivity.

The un-infected cell line (of each type) was treated as negative control in all of the cytotoxicity tests.

Statistical analysis

Quantitative numbers were expressed as means. Qualitative data were categorized as either positive or negative. The Minitab software was employed for determination of Pearson correlation, Odd ratio, Chi- square and P- value. The P- values of less than 0.05 were considered significant. For WST-1 the one-way ANOVA and the post-Hoc turkey methods were applied to the results.

Results

The results of bacterial biochemical tests are summarized in table 1. The rates of SOF+, Protease+, Lipase+ and DNase+ among tested strains were 75%, 56%, 88% and 88% respectively.

Fig. 1 demonstrates the internalized bacteria in A549 cell line and Fig. 2 (A→d) shows the CPE of each type of the infected cell line. Fig. 3(A→D) is a part of the results of DNA electrophoresis for the dead cells. The results of enzymatic biochemical tests are gathered in table 1. The data within table 2 is consisted of the results of the cytotoxicity of all strains using Trypan blue and electrophoresis. Although the quantities of three cytotoxicity methods are not exactly comparable, for ease of assessment, the percents of Trypan blue stain and WST-1 have been changed to positive or negative (compare

	Trypan blue staining				Gel electrophoresis				WST-1			
Strains	A549	BT-20	L-929	PC-3	A549	BT-20	L-929	PC-3	A549	BT-20	L-929	PC-3
M_1	✳ +	+	+	+	¤ +	+	+	+	¥ +	+	+	+
M_3	+	+	+	+	+	+	+	-	+	+	+	+
b300	+	-	-	-	+	-	-	-	+	+	+	+
d72	+	+	+	+	+	+	+	+	+	+	+	+
d65	+	+	+	+	+	+	+	+	+	+	+	+
d21	-	+	-	-	-	-	-	+	+	+	+	+
p13	-	+	+	-	+	+	+	+	+	+	+	+
n96	-	-	-	-	-	-	-	-	+	+	+	+
ganji	-	-	-	-	-	-	-	-	+	+	+	-
E. faecalis	-	-	-	-	-	-	-	-	+	+	+	+

✳ Positive by Trypan blue staining: the % of stained cell > negative control
¤ Positive by electrophoresis: formation of smear from DNA electrophoresis
¥ Positive for WST-1: the OD1- OD0 of infected cell line > un-infected cell line

Table 3. Comparison of the results of cytotoxicity of 9 strains of S. pyogenes + standard strain of E. faecalis on 4 types of malignant cell lines by 3 methods: Trypan blue, electrophoresis and WST-1 assay.

with negative control) in Table 3.

The correlation between Trypan blue stain and electrophoresis for 4 type's of cell lines: A549, BT-20, L-929 and PC-3- were 0.86, 0.63, 0.60 and 0.75($p<0.05$). But the correlation between Trypan blue staining and WST-1 was not significant ($p>0.5$) for A549 and BT-20 and ($p>0.05$) for L-929, it was o.73 ($p<0.05$) for PC-3.

In this study the electrophoresis of DNA displayed the basic method for evaluation of cytotoxicity. Detailed data on the subject of optical densities (OD1-OD0) and standard deviations (SD) in WST-1 are included in table 4. Each strain was tested for three times in parallel.

Discussion

Bacteria are rich sources of natural products. Some have attracted the attention of the drug industry as potent protective agents against natural threats like infection and cancer [29].

Historically, Streptococcus pyogenes has opened a new window on cancer treatment since two German physicians W. Busch and F. Fehleisen -separately- found that certain types of cancers regressed following streptococcal erysipelas in hospitalized patients [3].

The bacterium Streptococcus pyogenes is not considered a significant intracellular pathogen like Listeria or Shigella however, it can efficiently enter a variety of mammalian cells [6,7]. The S. pyogenes releases a wide range of lytic

	A549		BT-20		L-929 PC-3			
Strains	OD_1-OD_0	SD	OD_1-OD_0	SD	OD_1-OD_0	SD	OD_1-OD_0	SD
M_1	0.457	0.115	0.985	0.242	0.393	0.076	1.01	0.393
M_3	0.561	0.265	0.192	0.249	0.354	0.069	1.184	0.237
b300	0.509	0.144	1.360	0.208	0.379	0.085	1.322	0.168
d72	0.607	0.188	1.232	0.293	0.397	0.072	1.320	0.163
d65	0.561	0.206	1.221	0.375	0.377	0.093	1.275	0.140
d21	0.557	0.252	1.195	0.260	0.386	0.072	1.291	0.216
p13	0.529	0.210	1.205	0.230	0.396	0.069	1.477	0.385
n96	0.482	0.150	1.215	0.231	0.378	0.116	1.363	0.196
ganji	0.540	0.165	1.285	0.323	0.472	0.088	1.166	0.226
E.faecalis	0.520	0.130	1.265	0.236	0.475	0.092	1.221	0.191
‡ Control	1.136	0.177	1.867	0.261	0.766	0.099	1.747	0.392

‡ Un-infected cell line was tested as negative control

Table 4. The mean of difference of optical densities (OD1-OD0) and standard deviations (SD) in WST-1. Every strain was tested three times on triple cultures of each cell line.

enzymes /toxins e.g hemolysin and erythrogenic toxin [30]. Therefore the cellular death- as a consequence of streptococcal tissue infections- could be the outcome of bacterial product's activity and/ or induction of apoptosis in the infected cell [7].

Among more than 80 M types of S. pyogenes, the M3 has been recognized as the most invasive strain [31]. This type was employed as the positive control.

In the present study, the results of three cytotoxicity methods were not equal. For the establishment of cell death (apoptosis or necrosis), the electrophoresis of DNA was the method of choice [7, 22, 23], hense the results of two other methods were compared with this technique. Accordingly we got similar results from Trypan blue stain and DNA electrophoresis for 60 to 86% of the tests. Simplicity is the best known criterion of Trypan blue stain, but the technique might be accompanied by a high human error [32].

The cell viability was also determined by WST-1 reagent. The rate of positive result was 97% including for negative control (table 3). For one out of four cell lines (PC-3), the correlation of Trypan blue stain and WST-1 was significant ($p < 0.05$). The colorimetric WST-1 assay indicated the early cell-damage resulting from mitochondrial disorder [33, 34]. This assay has been employed in a few similar experiences [22] however we do not recommend it for tests where two types of viable cells (bacteria and eukaryotic cells) are involved.

Isolates of S. pyogenes induced various level of cell death (Table 2). In Bennett study (Australia- 1999), those isolates that recovered from invasive cases of S. pyogenes diseases, invaded HEp-2 to a significantly lesser extend than those from superficial sites [32]. In other study it was shown that the invasion of epithelial cells may not be related to the invasive disease of S. pyogenes [36].

Moreover, each types of malignant cell lines,

affected variably by streptococcal and even M3 invasions. The DNA electrophoresis, results indicated that the human prostate epithelial cell (PC3) resisted against M3 but this was not confirmed by Trypan blue nor by WST-1assay (Table 2 and 3).

Experimental investigations have indicated that, invasion of S. pyogenes is under the influence of streptococcal exoenzymes [10, 22, 37]. According to the results of the present study, the correlation between SOF production, lipase production, DNase production and cytotoxicity of S. pyogenes was not significant ($p > 0.05$, odd ratios: 0.37, 2.19 and 2.5 respectively) . However 67% of protease positive strains were cytotoxic, and the correlation of these characters was 0.78 by Trypan blue stain ($p< 0.05$, odd ratio: 11.37). For S. pyogenes protease positive, is linked to the production of streptococcal pyrogenic exotoxin (SPE) [7,19 ,22, 23]. The Spe B, significantly mediates the enhancement of invasion [7,19,38], when compared with other types of SPEs (Spe A, and Spe C)

Cancer treatment is encountered by various significant problems [1]. Bacteria could unlock secrets that aid cancer treatment [29]. In this way, a number of familiar human pathogens like Salmonella typhimurium, E.coli, Clostridium novyi, Pseudomonas aeroginosa, Streptomyces spp and their natural products have manifested excellent results [2,29,39,40].

The findings of present ex-vivo study showed that some S. pyogenes as a cusative agaent of benign infection like pharyngitis can effectively induce cell death in some carcinoma cell lines(within 24 hrs). It seemed that among streptococcal products, the proteolytic enzymes related to Spe-B - were more associated with bacterial invasion.

Hopefully it will be possible to design recombinant anti-cancer invasive bacteria by liganding to specific malignant cell receptors in the near future.

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