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RESEARCH ARTICLE

Invitro cytotoxicity study on recombinant human insulin produced in *pichia pastoris*

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Abstract

Background: Recombinant DNA technology has made a revolutionary impact in the area of human healthcare by enabling mass production of safe, pure and effective rDNA expression products, currently several categories of rDNA products like hormones of therapeutic interest, haemopoietic growth factors, blood coagulation products, thrombolytic agents, anticoagulants, interferons, interleukins and therapeutic enzymes are being produced using recombinant DNA technology for human use.

Materials and Methods :Production of recombinant human insulin was perfumed by standard methods total RNA was isolated from pancreatic tissue, then RNA was converted to cDNA using RT-P RCR reaction and preproinsulin gene was amplified using specific primers and inserted into the *EcoRI and Not*I sites of pPIC9K to produce the expression plasmid named *pPIC9K*-hpi expression vector . Which then transferred to *Pichia pastoris* by electroporation method? MTT assay and hemolysis test were carried out to evaluated the *invitro* cytotoxicity of recombinant human insulin that expressed into *Pichia pastoris* .

Results*:* the results demonstrate that the different tested concentrations of recombinant human insulin showed no inhibitory effect on the metabolism activity and cell viability of the human blood lymphocytes after a 24 h exposure, furthermore the result shown that the human insulin had no hemolytic activity against the human red blood cells within a different concentration used.

Conclusions: overall results amply demonstrate that the human insulin produced in this work is safe and had no cytotoxicity activity as the standard formulated insulin and recommended as hypoglycemic medical drug.

Introduction

Recombinant DNA technology has made a revolutionary impact in the area of human healthcare by enabling mass production of safe, currently several categories of rDNA products like hormones of therapeutic interest, haemopoietic growth factors, blood coagulation products, thrombolytic agents, anticoagulants, interferons, interleukins and therapeutic enzymes are being produced using recombinant DNA technology for human use. (Uttam *et al*., 2013). A classical example of recombinant DNA technologies successful use is in the production of human insulin for the treatment of *diabetes mellitus*. The availability of human insulin as the first pharmaceutical products produced in *E. coli* and manufacturing through the recombinant DNA technologies was launched in the market in 1982 But with its increasing demand, efforts to develop more cost-effective production schemes have not diminished **(**Johnson *et al*., 1983). The earliest insulin preparations were obtained from beef pancreas. They were unstable in neutral solution and were provided to patients in powder or tablet form, which was suspended in water or

saline immediately before injection. Stable amorphous preparations in acid solution were then developed (Rolf *et al*., 2014). Genetically engineered human insulin is most commonly used and now the vast majority of people requiring insulin treatment worldwide are prescribed synthetic human insulin (Zalinah *et al*., 2014).

Now day human insulin is produced by recombinant DNA technology and various companies differ in their methodology but the basic principle is the introduction of human insulin gene into microorganisms which keep on multiplying and in turn producing insulin such as yeast. (Joshi *et al.*, 2007). Yeast is well suited to the expression of heterologous proteins of pharmaceutical importance. Yeast's secretory pathway exhibits much of the structure and function of the mammalian secretory system and has the capacity to fold, to process proteolytically, to glycosylate and to secrete protein (Thomas *et al*., 1999).

The safety assessment of drugs derived from recombinant-DNA microorganicims involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on human safety. Different techniques were used to measure changes in cell viability and inter actions with erythrocytes. In this study, we examined the in vitro cytotoxicity of recombinant human insulin by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and hemolysis test to determine detrimental intracellular effects on mitochondria and metabolic activity (Choksa *et al*., 1995).

2. Materials and Methods

2.1. Total RNA Extraction from Human Pancreatic Tissue

Normal human pancreatic tissue of a brain death patient was provided from the cancer center for organ recovery and education resource (C.O.R.E). Fudan medical university, Shanghai, and immediately frozen in liquid nitrogen and then kept at -80˚C. Total RNA was extracted using the total RNA extraction kit (QIAGEN) under native condition and according to manufacturer's instructions . RNA concentrations were measured using DU-640 nucleic acid and protein analyzer (backmen,USA). RNA integrity was evaluated by visualization of discrete 28S, 18S and 5S rRNAs through 1% agarose gel (Sambrook and Russel., 2001).

2.2. The amplification of human preproinsulin gene

RNA Samples of good quality were chosen for reverse transcription reaction (cDNA synthesis) , by using Reverse .Transcription System kit **(**Promega, USA) according to the manufacturer's instructions. In a final volume of 20 µl , reaction mixtures was prepared by mixing the following component (MgCl2, 25mM 4 ml , Reverse Transcription 10X Buffer 2µl ,dNTP Mixture, 10mM 2µl , Recombinant ribonuclease inhibitor 0.5µl, AMV Reverse Transcriptase15 u (High Conc.) , Oligo(dT)15 Primer 0.5µg, Total RNA 7µl) by vortex for 20 sec and centrifuged to precipitate the components. The reaction mixture was incubated at 42 °C in water bath for 15 minutes, then the sample was heated at 95 °C for 5 min and incubated at 0 - 5 °C for 5 min .The first strands cDNA was transferd to new RNase free micro centrifuge tube and then stored at -20°C until further used.

The amplification of human preproinsulin gene was carried out by using the first-strand cDNA as a template using non - proofreading thermostable DNA polymerase, Nova *Taq*. DNA polymerase, and two sets of specific primers were used for human preproinsulin gene the forward primer for preproinsulin gene was 5' GGGGAATTC ATTAATTCGCGGCCGCATGATGA 3' and the reverse primer was 5' TTATTGCGGCC GTGACGACGAATTCTTTGTCAACCAACA3' Primers contained *EcoR1* and *Notl* restriction sites on the 5`ends according to (Rohde, 2006) for amplification of human preproinsulin fragment.

2.3 Construction of human preproinsulin into expression vector:

The modified *pPIC9K* expression vector used for ligation of *hpi* gene has been provided from invetrogen company , the vector employed for this study containing the *a*-factor secretion signal directed release of the recombinant protein into the culture supernatant media The vector and the PCR amplified gene was double restricted with *EcoRI* and *Not 1* enzymes. Both the restricted products were cleaned up using (*Axygen*) gel clean up kit. The product obtained after clean up is checked on 1 % agarose gel for sizes, and ligation reactions was carried out by inserted the *hpi* into the *EcoRI and Not*I sites of pPIC9K to produce the expression plasmid named *pPIC9K*-hpi expression vector .

2.4 Transformation of *Pichia pastoris*

Pichia pastoris GS115 was used for the transformation with recombinant plasmid by electroporation method as described by (Scorer**,.** et al 1994) Cells were electroporated using an electroporator gene pulser apparatus (Bio-Rad, Hercules, CA USA) with the following settings: High-voltage mode, resistance (200 Ω) Ohms, charging voltage of 2.5 kV/cm and pulse length of 5 msec. according to the producer's instruction. The Plasmid integration in transformants was verified by extraction genomic DNA of *Pichia pastoris* and used as a temple for PCR reaction with two set of primers forward primer GACTGGTTCCAATTGACAAGC(5'-*AOX*) and reverse primer GCAAATGGCATTCTGACATCC (3*'AOX)* , to confirm the integration of recombinant vector with *hpi* gene into *P. pastoris* genome according to (Linder *et al*. 1996).

2.5 Protein expression and purification

Protein expression was assessed by performing small scale expression studies using a single transformants colony able to grow on MD plates without histidine supplement was patched using a sterile toothpick and inoculated in buffered methanol-complex medium BMMY, The flask was incubated at 30°C in a shaking incubator at 220 rpm for 72 hr, The cultures were supplemented with 1% methanol every 12 h in order to induced *AOX1* promoter after inoculation . The insulin protein expressed in cultures was confirmed by using Tricine - Sodium dodecyl sulfate polyacrylamide gel electrophoresis, according to the method described by (Schagger and von Jagow, 1987). and Western blot analysis as described by (Bjerrum et al 2001). Crude extracts obtained from induced cultures grown for 72h in BMMY medium as previously described was fractionated by precipitation with ammonium sulfate((NH4)2SO4) , according to the procedure described by (Green and Hughes 1955). The resulted Protein extracts was purified under native conditions using the glass chromatography column on Ni-NTA Purification system from (Qiagen, CA) according to the manufacturer's instructions.

2.6 Evaluation of recombinant human insulin cytotoxicity using the MTT assay

The MTT (3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyl tetrazolium bromide) assay was used to determine the *in vitro* cytotoxicity of recombinant human insulin against human blood lymphocytes, based on the selective ability of viable cells to convert MTT into purple formazan by mitochondrial dehydrogenase enzymes relies on intact metabolic activity and is frequently used for screening of cytotoxicity as described by (Mosmann., 1983). In the present work pure human blood lymphocytes cells were isolated from 5ml of Blood samples collected from healthy volunteers using ficoll density gradient technique described by (Boyum., 1968). Lymphocytes were prepared in serum supplemented tissue culture medium (RPMI 1640+FCS 10%) and sterilized by 0.2 mm filtration, then lymphocytes cells (100 µl/well) were seeded at seeding densities of 1x105 cells/ml into 96 well microtitre flat – bottomed plates and allowed to adhere for 24 h. at 37 °C in the presence of five different concentrations of recombinant human insulin solutions (50,100,150,200, and 250µg/ml). After 24 h of exposure, medium was removed and 100 µl of growth medium with sterile filtered MTT (5 mg/ml in PBS) was added to each well. Cells were incubated at 37 °C in humidified atmosphere for 4 hr, after that medium was removed by aspiration and the insoluble formazan crystals were dissolved by added 100 µl 0.1% DMSO dimethylsulfoxide (Merck, Darmstadt, Germany) to each well with shaking gently until the crystal was completely dissolved, then incubation at 37 ºC for 30 min. finally the absorbance was measured in an ELISA plate reader at 540 nm. , absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only (as control) were taken as 100 % cell viability

2.7 Evaluation of recombinant human insulin cytotoxicity using hemolysis test

In vitro hemolysis testing was designed to screen the hemolytic activity of recombinant human insulin against human red blood cell (R.B.C) as recommended by the US FDA, and according to the procedure described by (Nair *et al* 1989). In the present work 5ml blood samples from healthy volunteers was collected into vials with heparin as anticoagulation according to the national institutes of health and drug administration the time and date of blood collection was recorded directly on the vial , and the blood was used within 6 hours of collection .Blood collected in heparinized-tubes was centrifuged at 1000 rpm for 10 min, the pellet was washed three times with cold PBS pH 7.4

by centrifugation at 1000 rpm for 10 min and resuspended in the same buffer. Recombinant human insulin solutions of different concentrations (10, 50,100 and 200 ppm), also was prepared in the PBS buffer, then the solution was exposed to the erythrocytes and were incubated for 60 min at 37C in a shaking water bath. The amount of free release hemoglobin of the supernatant was measured by ELISA at 540 nm, after centrifugation at1000 rpm for 10 min. Complete hemolysis was achieved using 2% Triton X-100 yielding the 100% control value. Less than 10% hemolysis was regarded as non-toxic effect level in our experiments. The experiments were run in triplicate and were repeated twice.

3. Results

Total RNA was extracted successfully by trizol method from normal human pancreatic tissue the results showed that total RNA was isolated successfully by trizol method from the pancreatic tissue and exhibit clear bands of 28S and 18S.

RNA samples good quality isolates from the pancreatic tissue has been used as template for complementary DNA (cDNA) synthesis the results of amplified individual gene shows the presence of a clear cDNA band of size approximately 330bp. in comparison with the standard molecular DNA ladder (2000bp. - 100 bp),

in the agarose gel electrophoresis as shown in fig (1) . The detected band in this region confirms the PCR reaction is carried out correctly. Furthermore the result of construction amplified PCR of human preproinsulin gene fragment into *pPIC9K* vector showed the presence of a sharp band of about 9. 630 bp just below the 1 kb band of the DNA ladder as shown in Figure (2) lane 3the presence of such band conform the size of *pPIC9K P. pastoris* expression vector after ligation as the size of vector is about 9300 bp (Invitrogen 2010).

On other hand the result indicated that *Pichia pastoris* GS115 was successfully transformation with recombinant plasmid by electroporation method and the protein was expressed successfully and presence as a single major band with about (5.8 KDa) these bands correspond well with the size of human insulin with the theoretical molecular weight (5.800 kDa). The observed results was confirmed by the presence of the expressed recombinant protein in the same location on western blot PVDF membrane of approximately 5.8 kDa the size of this band corresponds well to the calculated size of human insulin -protein (5.88kDa) (Gualandi ., *et al* 2001). As shown in Figure (3).

In this study MTT assay was used to determine the degree of *in vitro* cytotoxicity of recombinant human insulin against human blood. Results demonstrate that the different tested concentrations of recombinant human insulin showed no inhibitory effect on the metabolism activity and cell viability of the human blood lymphocytes after a 24 h exposure, In contrast SDS, the positive control significantly reduced human blood lymphocytes cell viability at a concentration of 0.1% , The different tested concentrations of recombinant human insulin were 50,100,150 ,200 and 250 µg/ml produces 97.8% , 95.6 %, 94.3% , 93.3% ,and 91.9 % cell viability respectively as shown in Figure(4) .

Furthermore the *in vitro* hemolysis testing was designed to screen the hemolytic activity of recombinant human insulin against human red blood cell (R.B.C) as recommended by the US FDA. Recombinant human insulin solutions of different concentrations (10 – 200 ppm) was exposed to the erythrocytes and were measured by ELISA reader at 540 nm. The result shown that the human insulin had no hemolytic activity against the human red blood cells within a different concentration ranging used, in compare with red blood cells treated with gradient concentration 0.01% , 0.1% v/v and 1% v/v of Triton X-100 that showed dose-dependent effect of hemolytic activity as shown in table 2 and figure (5)

330 bp

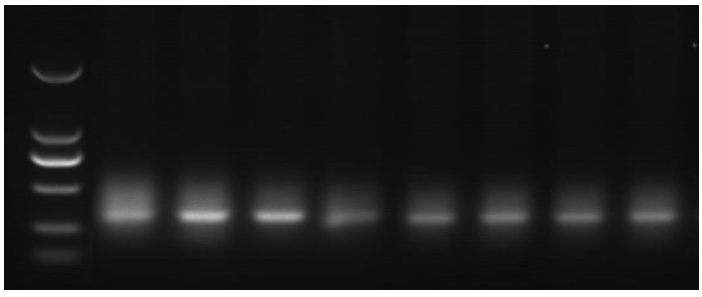
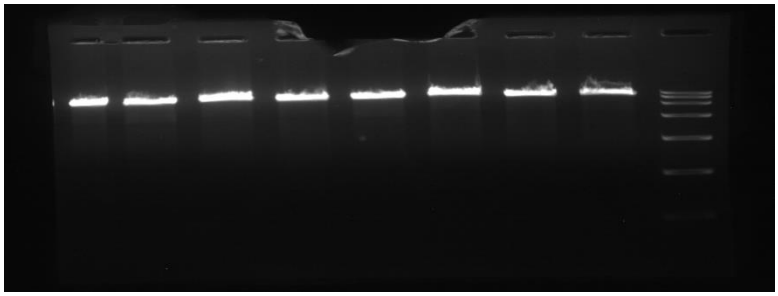
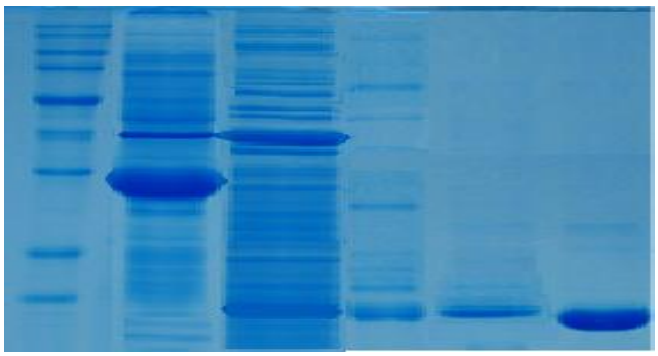


Figure 1: The analysis of 1.5 % agarose gel electrophoresis for c DNA isolates from pancreatic tissue sample. Lane1: DNA marker 2000 Bp (100; 250; 500; 750; 1000; 2000). Lane 2: c DNA

9 630 bp



Fig(2) : The analysis of 1% agarose gel electrophoresis with the expression constructive vector *pPIC9K - hpi* (9.630 bp.) Lane 1: DNA marker 15000 bp (250; 1000; 2500; 5000; 7500; 10000 ; 15000) Lane: 2, 3,4,5,6 : *pPIC9K* expression vector .



5.800 KDa

Figure (3) : SDS-PAGE analysis of the Ni-NTA purification of recombinant human insulin

(lane 1)Standard molecular weight protein marker (6.5; 14.3; 20.1; 29; 44.3; 66.4 ; 97.2; 116; 200 KDa).
(lane 2) the flowed through Proteins
(lane 3) The lysate
(lane 4) Column washed with 50 mM imidazole to remove non-specific binding proteins
(lane 5) Column washed with 100 mM imidazole to remove non-specific binding proteins
(lane 6) eluted of the recombinant proteins with 200mM imidazole

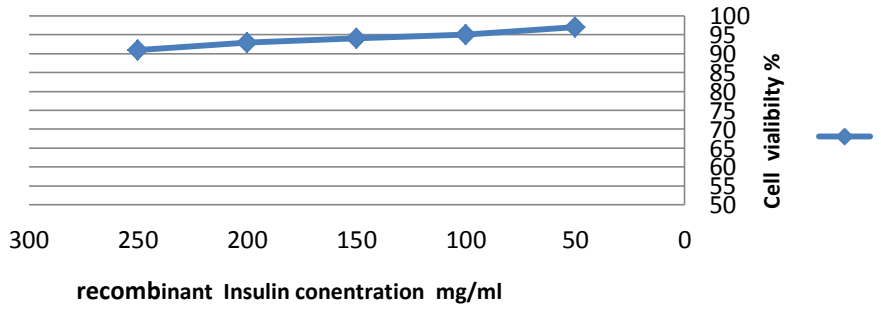


Figure (4)Cytotoxici activity of recombinant insulin against human lymphocytes cells as measuered by MTT assay

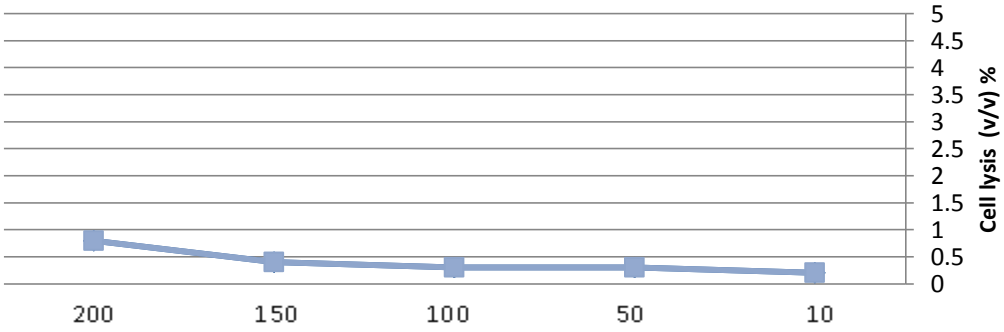


Figure (5) Hemolytic activity of recombinant human insulin against human red blood cells.

4- Discussion:

Many proteins such as human preproinsulin cannot be obtained from their natural sources in suitable quantities because of their low abundance or difficulty of purification by conventional methods from human tissue samples, organs or cell lines. To find the solution for this problem, production of recombinant human proteins in heterologous eurokaryotic cell systems such as yeast was suggested. The methylotrophic yeast *Pichia pastoris* is one of the major eukaryotic expression systems and extensively used for the production of recombinant proteins. Due to the possible humanization of its glycosylation pattern (Alexandra, 2010).

In the present work the cytotoxicity effect of different concentrations of recombinant human insulin was evaluated against metabolism activity of human blood lymphocytes by using the MTT assay. Several recent researches were use MTT assay to evaluate the cytotoxicity effect of pharmaceutical protein produced by recombinant DNA technology (Davi *et al*., 2014 ;Zhang *et al*., 2011; Ziegler *et al*., 2008). Recombinant human insulin Produced in this work showed no inhibitory effect on cellular metabolism activity and cell viability at concentrations up to the maximum concentration used (250 µg /mL) after a 24 h exposure, in contrast, SDS the positive control as illustrated in Figure (20.4). Cytotoxicity is the harmful or noxious unwanted effect induced by a biomaterial in cell culture system *in vitro*. During the last years, the interest of *in vitro* systems, as an alternative to animal experiments in toxicological research has been steadily increasing (Ciapetti *et al*., 2005). Our data demonstrated that the different tested concentrations of recombinant human insulin were 50,100,150 ,200 and 250 µg/ml produces 97.8% , 95.6 %, 94.3% , 93.3% ,and 91.9 % of cell viability respectively and showed no significant differences ($P > 0.05$) when compared to that of negative control.

In contrast of SDS, the positive control that showed significantly reduced ($P < 0.05$) on human blood lymphocytes cell viability at a concentration of 0.1%. MTT is a yellow water-soluble tetrazolium dye which is metabolized by the mitochondrial living cells to a purple formazan product insoluble in aqueous solutions. The amount of formazan generated is directly proportional to the number of viable cells (Johan *et al*., 2005). in our assay ,no difference were observed with regard to the sensitivity of the blood lymphocytes cells for the Produced human insulin ,as the sensitivity is crucial to measure intermediate toxic response (*Park et al*., 2005). And appearing of the purple color in the lymphocytes culture exposure to the produced insulin indicated that MTT dye was metabolized by these cells, in this in turn indicating that there was no inhibitory effect on cellular metabolism activity and cell viability (Susan *et al*., 1992).Based on results obtained here we can conclude a preliminary demonstration of the safety *use* of this produced insulin as a *medical* product .

To confirm the results of MTT assay against produced recombinant human insulin hemolysis test was perfumed. The hemolysis test is an important measure of the ability of a material or material extract to cause red blood cells to rupture. Hemolysis testing should be performed on all materials directly contacting the bloodstream, or any materials used to form a fluid conduit to the bloodstream. The following tests are derived from well-

established studies/standards and are useful in evaluating a variety of materials intended to contact blood or fluids entering the circulatory system. (Samoul, 2002).

The *in vitro* hemolysis testing was designed to screen the hemolytic activity of recombinant human insulin solutions of different concentrations (10 ,50,100and 200 ppm) against human red blood cell (R.B.C) as recommended by the US FDA .

The result shown that the human insulin had no hemolytic activity against the human red blood cells within a different concentration ranging used from 10 - 250 ppm, in compare with red blood cells treated with gradient concentration 0.01% , 0.1% v/v and 1% v/v of Triton X-100 that showed dose-dependent effect of hemolytic activity as shown in table 3.6 and figure (19.4) .

in this experiment the amount of free hemoglobin release into suspension cells that exposure to the produced insulin at gradient concentration (10 ,50,100and 200 ppm) was very low and this was in turn reflected the percentage of hemolytic activity 2%,3%,5%, and 8% hemolysis ,as the amount of free hemoglobin release into plasma is an indicator of red blood cell lysis (Jeanete *et al*., 2005) . And all these percentage is Less than 10% hemolysis and was regarded as non-toxic effect level in our experiments (FAD 2005) .overall results amply demonstrate that the human insulin produced in this work is safe and had no cytotoxicity activity as the standard formulated insulin and recommended as hypoglycemic medical drug.

Statistical analysis:

The MTT analyses were performed using Student's *t*-test for unpaired data. *P* values < 0.05 were considered statistically significant.

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