**BIOACTIVITY OF RECOMBINANT HUMAN INSULIN PRODUCED BY 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 human insulin was perfomed by standard methods total RNA was isolated from pancreatic tissue, then RNA was converted to cDNA using RT-PCR reaction and preproinsulin gene was inserted into the pPIC9K to produce the expression plasmid named pPIC9K-hpi expression vector. Which then transferred to *Pichia pastoris* by electroporation method. four groups of diabetic rats were used in this study according to protocol used by animal models of diabetic complications consortium (AMDCC) members to induce type 1 diabetes in a number of the animal models developed by the consortium (AMDCC, 2013). Plasma glucose levels of all four groups were measured before and after experimentation.

**KEY WORDS:** recombinant human insulin, pichiapastoris, antidiabetic activity, glucose levels.

**1. INTRODUCTION**

The earliest insulin preparations were extracted 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). In the early 1980s, human insulin was prepared in recombinant bacteria and this protein which proved of better quality than the conventional insulin, recombinant human insulin is almost exact to natural human insulin, and has almost completely replaced animal insulin because animal insulin is slower rate of absorption into the bloodstream. Its cause an Immunological complications, allergy has been particularly common, with local symptoms still occurring in ~5% of all patient, it is less effective, and it is expensive to produce (Narendran et al., 2005). Since this time recombinant human Insulin remains the most effective and consistent means of controlling blood glucose levels in all patients suffering from diabetes (David, et al. 2014).

The use of recombinant DNA technology has enabled the production of large quantities of protein drugs as cost-efficient sources, these proteins perform the same function as naturally occurring proteins in body (Reza et al., 2011). Many proteins such as human insulin 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 (Bruce et al., 2013). In present, three major methods have been used for the production of human insulin in microorganisms, the first method involve Escherichia coli with the expression of a large fusion protein in the cytoplasm. The second methods involve the use of a signal peptide to enable secretion into the periplasmic space also in Escherichia coli A third method utilizes the Saccharomyces cerevisiae to secrete the insulin precursor into the growth medium (Terry et al. 2011). 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 (Daniel et al., 2013).

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 Russell. 2001).

2.2. The Amplification of Human Proinsulin 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 mixtuer 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 transferred to new RNase free micro centrifuge tube and then stored at -20°C until further used. The amplification of human proinsulin gene was carried out by using the first-strand cDNA as a template using non-proofreading thermos table 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 ATTAATTCGCGGCGCATGATGA 3' and the reverse primer was 5'TTATTGCGGCC GTGACGACAATTCTTTGTCAACCAACA3' Primers contained EcoR1 and NotI restriction sites on the 5’ends according to (Rohde, 2006) for amplification of human preproinsulin fragment.

2.3 Construction of Human Proinsulin into Expression Vector
The modified pPIC9K expression vector used for ligation of hpi gene has been provided from invitrogen 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 doublerestricted with EcoRI and NotI enzymes. Both the restricted products were cleaned up using (Axygen) gel clean up kit. The product obtained after cleanup is checked on 1 % agarose gel for sizes, and ligation reactions was carried out by inserted the hpi into the EcoRI and NotI sites of pPIC9K to produce the expression plasmid named pPIC9K-hpi expression vector.
2.4 Transformation of Pichiapastoris

*Pichiapastoris* GS115 was used for the transformation with recombinant plasmid by electroporation method as described by (Scor, et al. 1994). Cells were electroporated using an electroporator gene pulsar 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 transform ants was verified by extraction genomic DNA of *Pichiapastoris* and used as a temple for PCR reaction with two set of primers forward primer GACTGGTTCCAATGTGACAAGC (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 transform ants 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.

2-10-3-2 Diabetes Induction

Twenty of adult Westar rats male 60 days old and weighed 250-300 gm, was used as animal models of diabetic complications consortium according to (AMDCC) members to induce type 1 diabetes in a number of the animal models developed by the consortium (AMDCC, 2013). After two weeks of acclimatization period westar rats male were made diabetic by given a single dose intraperitoneal (i.p.) injection of streptozotocin . Each rat received 50
mg/kg body weight of STZ solublized in 0.1 M citrate buffer for 12 days to induce diabetes. Control rats were injected with citrate buffer solution alone, uninjected rats of comparable ages served as control group. Blood glucose levels were examined 14 days after the final streptozotocin injection by obtaining blood from the tail vein and measuring glucose concentration with a glucometer (One Touch Accu-Check instant, USA). The animals chosen as diabetic were those which presented fasting glycemia above 200 mg/d, at the end of the experiments, the animals were weighed; the rats were equally divided into four groups of 5 rats each, given the following treatment:

1. **Group 1** (Diabetic rats): this group included 5 diabetic rats, were injected subcutaneously by recombinant human insulin 100 µl /100 g body weight of animal for 3 days.
2. **Group 2** (Diabetic rats): this group included 5 diabetic rats, were injected subcutaneously by Lente insulin (Eli Lily and Co., Indianapolis, IN), 2 units/100 g body weight for 3 days.
3. **Group 3** (control): this group included 5 diabetic rats, this group were orally administrated with 5ml normsaline, this group was used as diabetic control.
4. **Group 4** (normalrats): this group included 5 rats of normalrats were used as normal animals.

2-10-3-3. Measurement of Glucose Levels.
Normal and diabetic blood samples of experimental rats were collected from rats in order to measure glucose levels, blood samples were collected from the heart of the rats after sacrificed and then, their blood was collected by using 1 ml insulin syringe and transferred into sterilized test tubes. The time of harvested was recorded and kept at 4 °C, and allowed to clot at room temperature for 5 min, then the serum was separated by centrifuged at 4,000 rpm for 10 min, the glucose levels were measured enzymatically by using Abcam’s glucose assay kit colorimetric assay according the manufactures ’s instructions. Serum glucose reactenzymitically with a dye and generate color at zero time, 2 hr, 4 hr, 6 hr, and 24 hr. Products was read at 570 nm. (AL-Shamonyet al.,1994).

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 exhibitclear bands of 28S and 18S. RNA samplesgood 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. 700 bp just below the 1 kb band of the DNA ladder as shown in Figure (2) lane 3 the presence of such band conform the size of pPIC9KP. Pasteoris expression vector after ligation as the size of vector is about 9300 bp (Invitrogen 2010) on other hand the result indicated that Pichiapastoris 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 (6 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 PVD F membrane of approximately 58 kDa the size of this band corresponds well to the calculated size of human insulin -protein (588kDa) (Gualandi., et al 2001). As shown in Figure (3).

The effect of product human insulin on blood glucose levels
Plasma glucose levels of all four groups were measured before and after experimentation. Normal levels of glucose in healthy adult rats were measured as 125±5 mg/dl. And the water and food consumption per day were measured as 40±5 ml and 15±2 gm, respectively. But in STZ-induced diabetic rats the levels of glucose, were measured as 450±20 mg/dl, and daily consumption of water and food in them were measured as 120±5 ml, and 50±4 gm, as Shawn in figure (4). and (5).

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
Fig(2) : The analysis of 1% agarose gel electrophoresis with the expression constructive vector pPIC9K - hpi (9.700 bp.) Lane 1: DNA marker 15 000 bp (250; 1000; 2500; 5000; 7500; 10000; 15000) Lane: 2, 3, 4, 5, 6 : pPIC9K expression vector.

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 KD)
(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
The results show significant variability between the 4 groups of Whistar rats in their fasting blood glucose levels, and there was a progressive increase in the blood glucose level of STZ-treated rats after each day of injection with STZ (126.0, 182.3, 243.7, 315.4 and 385.7 mg/dl, respectively), resulting in a significant difference from that of the control group rats (120.5, 135.2, 131.5, 126.8, and 132.2 mg/dl, respectively), as shown in figure (6).
From other hand ,the results show a dose-dependent lowering significant effect on serum glucose levels by both insulins (human produced insulin and stander insulin) was observed in STZ-induced diabetic rats groups at the end of 240 minutes of the injection (p < 0.001) , in compared with untreated group STZ-induced diabetic rats ,as Shawn in table (7). The recombinant human insulin was found to have acute effects on decrease the blood glucose level. It was noticed that the produced insulin resulted in reduction of blood glucose level of 385.7 to 136.0 mg/dl was observed in STZ-induced diabetic rats groups at the end of 240 minutes, while the standard insulin resulted in reduction of blood glucose level of 395.6 to 115, 8 mg/dl was observed in STZ-induced diabetic rats groups at the end of 240 minutes as Shawn in fig (7). Significantly decreased the blood glucose level in these animal suggesting that it has antidiabetic properties in a dose dependant manner.

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 eukaryotic cell systems such as yeast was suggested. The methylotrophic yeast Pichiapastoris 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), the results show a dose-dependent lowering significant effect on serum glucose levels by both insulins (human produced insulin and stander insulin, Humulin R, by Elli Lilly and Co.) was observed in STZ-induced diabetic rats groups at the end of 240 minutes of the injection (p < 0.001), in compared with untreated group of STZ-induced diabetic rats , as Shawn in fig (7).
(7) acute effects on decrease of blood glucose level was observed (385.7 to 136.0 mg/dl), in STZ-induced diabetic rats groups that subcutaneously injected with human produced insulin at the end of 240 minutes of the injection. While the stander insulin, (Humulin R, by Elli Lilly and Co.) showed no significant differences (P > 0.05) when compared to that resulted in reduction of blood glucose level (395.6 to 115.8 mg/dl) in STZ-induced diabetic rats groups at the same period fig (7).

Hence proves that both insulins had the similar mode of action and activation of its cell-surface receptor to absorb the blood sugar and stimulate the peripheral glucose consumption (Sharma, and. Kumar. 2011). And both insulins had been absorbed in similar duration that means our produced insulin had the same pharmacokinetic and biochemical properties of the stander insulin, (Humulin R, by Elli Lilly and Co.) (Vijay et al., 2014; Takahisa et al., 2014) Overall result we can concluded that the significantly decreased the of blood glucose level in these diabetic animal suggesting that this insulin has anti-hyperglycaemic properties and have potential to reduce serum blood glucose levels in a dose dependant manner and had no cytotoxic activity .it is evident that the insulin produced in this work is active as the standard formulated insulin and recommended as hypoglycaemic drug.

CONCLUSIONS

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