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In Vitro Effect of Insulin on *Acanthamoeba Spp.*

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Abstract
Acanthamoeba spp. is an opportunistic protozoa that causes a threat to the health of humans and animals due to its ability to develop inside and outside the host. It can cause serious and fatal infections in different places in the host's body, depending on the host's immune status and strain type. This paper aims to evaluate the lethal effect of insulin on Acanthamoeba spp. in vitro. Three concentrations (25, 50, and 100 IU/ml) were used to evaluate the lethal action of insulin at exposure periods (5, 10, and 15 minutes). The data were analyzed using SPSS and one-way ANOVA. The most potent lethal effect was recorded 15 minutes after exposure for all concentrations used, with survival rates of 44.90%, 31.90%, and 0%, respectively. Meanwhile, 5-minute exposure showed the lowest activity of 77.00%, 57.04%, and 12.00% at the concentrations used, respectively. The results of the in vitro study confirmed the effectiveness of insulin as an amoebicidal agent with increasing concentrations and exposure periods.

Key words: Acanthamoeba, insulin, amoebicidal, survival rate

Introduction

Insulin is the first peptide hormone discovered as it was isolated from the pancreas of dogs in 1921 by Canadian scientist Frederick Banting and Romanian physiologist Nicolas Paulescu. After isolating insulin, they began to obtain a purified extract, which was accomplished with the help of a Scottish physiologist, John Macleod, and a Canadian chemist, James Collip. Insulin is regarding the first hormone that was synthesized chemically (Weiss *et al*., 2000).

Insulin is a peptide hormone produced in the islets of Langerhans in the pancreas by beta cells. It is one of the main anabolic hormones. It regulates the metabolism of fats, carbohydrates, and proteins by stimulating glucose absorption from the blood into liver cells, skeletal muscles, and adipose tissue. It converts the absorbed glucose into these tissues through a synthesis process. Glycogen is converted into glycogen, or it is converted into fat in the liver during the process of manufacturing fats, or both. High concentrations of insulin in the blood inhibit the production of

glucose and its secretion through the liver (Weiss *et al.*, 2000). Secreted insulin also affects the synthesis of proteins in tissues. Therefore, insulin is considered an anabolic hormone, as it converts small molecules in the blood into large molecules inside cells. However, low levels of insulin in the blood have the opposite effect by stimulating catabolism at a significant level, especially fats stored in the body (Sonksen and Sonksen, 2000).

Insulin is secreted due to several factors, but the most important of them is the sensitivity of beta cells to high levels of glucose in the blood and vice versa; insulin secretion decreases when the glucose level in the blood decreases (Koeslag *et al*.,2003). Insulin secretion can also be stimulated by some amino acids, fatty acids, keto acids (fatty acid oxidation products), and many of the hormones secreted by the digestive system. Insulin secretion is inhibited by somatostatin and activating the sympathetic nervous system. Insufficient production of insulin leads to diabetes, which is a condition of high blood sugar levels. Insulin injections are given to patients with severe diabetes. Insulin injections were first used from cattle, pigs, and sheep hormonal extracts. Still, in the early 1980s, insulin was produced from the genetic modification of certain strains of bacteria to produce the human insulin hormone using recombinant DNA techniques (Weiss *et al*., 2000).

The human insulin hormone consists of 51 amino acids. It is divided into two chains: the A chain, consisting of (21 amino acids) and the B chain, consisting of (30 amino acids) linked to each other by three disulfide bonds, and its molecular mass is (5808) kilodaltons (Tokarz *et al*., 2018).

Insulin is one of the most widely studied hormones (Brange and Langkjoer, 1993). Still, the detailed facts about the origin and expression of proteins similar to the human proteins IR, IGF1-R, and GLUT4 in unicellular eukaryotic organisms have not been studied, as stated in a study (Baig and Khaleeq, 2020) conducted a study on *Acanthamoeba castellani*, which has been present in the environment for about two billion years (Cooper, 2000), where glucose balance and the possible expressions of proteins related to IR, IGF1, and GIUT were studied. They found that insulin affects the spread of the vegetative phase of the parasite.

Acanthamoeba is considered a widespread parasite that lives freely or opportunistically. It has also been isolated from various environments (Mahdi and Muslim, 2023). *Acanthamoeba* cause many diseases, such as amoebic keratitis, which threatens sight, and granulomatous amoebic encephalitis, which is fatal (Marciano-Cabral and Cabral, 2003), as it threatens human life and health. The parasite infection rate is considered low compared to the relatively high death rate for patients with high or low immunity (Lanocha-Arendarczyk et al., 2017).

It is known that *Acanthamoeba* grows in media containing glucose, such as PYG medium, which is rich in glucose for the growth of the vegetative phase of Acanthamoeba (Hameed, 2015), as the parasite consumes glucose by metabolizing carbohydrates and analyzing sugars into glucose for use (Kanehiea *et al*., 2016). This study aims to demonstrate the effect of the hormone insulin on the vitality of the parasite and determine the lethal dose for the parasite.

Materials and method

Isolation of *Acanthamoeba* parasite

Samples were collected from people suspected of being infected with *Acanthamoeba* keratitis at Basrah Teaching Hospital and Al-Sadr Teaching Hospital according to the task facilitation letter issued by the Basrah Health Department (Training and Human Development Center and Research Committee) No. 333 dated 12/19/2022. Samples were isolated from the eye using a sterile cotton swab, taken from the inferior conjunctiva by rolling the swab in a circular motion from side to side, and examined with a light microscope (Anisah *et al.*, 2005).

Cultivation Media

Peptone Yeast Glucose Agar (PYGA) medium was used for *Acanthamoeba* growing which prepared from [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), glucose 1.5% (w/v) and 1.0% agar], the pH of the culture medium was adjusted to 6.5- 6.6 by adding 1 M aqueous sodium hydroxide (NaOH), 0.1 mg/ml of penicillin was added, then the medium was sterilized and stored at 30-32 ^{o}C (Hameed, 2015).

To obtain sufficient vegetative and cyst stages of *Acanthamoeba* for this study, a large numbers of culture dishes were prepared. Non-nutrient agar medium (NNA) was also used to fix the parasite, which was prepared by dissolving 15 g of agar (Sigma-Aldrich) in 1000 ml of distilled water and then sterilizing the medium at 121 °C for 15 minutes (Muthu Kumar *et al.*, 2022).

Amoebicidal Assay

Our study examined the effect of different concentrations (25, 50, and 100 IU) of insulin against *Acanthamoeba* for 5, 10, and 15 min. 1 ml of the Amoeba solution (containing approximately 6*104 amoeba /ml) was placed in each test tube and 1 ml of the insulin was added in different concentrations to each test tube. The tubes were incubated for 5, 10 and 15 min at 37 °C; then, the supernatant was removed at the end of the incubation period. A volume of 1 ml of 0.1% trypan blue stain was added in order to test the Viability of the living amoeba, determining the number of live (non-stained) and dead (stained) cells. A drop was smeared on a glass slide, covered with a coverslip, and examined under a light microscope. The percentage of dead amoeba was counted by hemocytometer, and non-treated amoeba was used for controls. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate.

Statistical Analysis

Statistical analysis was undertaken using SPSS software version 24. A one-way ANOVA test was utilized to identify the differences between the control group and the test, and a value of less than 0.5 was considered significant.

Results

Insulin showed lethal activity within the periods and concentrations used in the study compared to the control group (P<0.05). The survival rate also decreased with increasing concentrations and exposure periods (Table - 1). The results of the study for a concentration of 25 IU/ml of insulin showed survival rates of 77.00%, 60.90%, and 44.90% with exposure periods of 5, 10, and 15 minutes, respectively. Using a concentration of 50 IU/ml of insulin showed survival rates of 57.04%, 45.30%, and 31.90% in periods of 5, 10, and 15 minutes, respectively. At the same time, a concentration of 100 IU/ml gave survival rates of 12.00%, 0.00%, and 0.00% after 5, 10 and 15 minutes, respectively. The highest concentration, 100 IU/ml, had the highest amoebicidal effect on the parasite (0%) after 10 minutes of exposure to this concentration.

Table 1: Survival rates for *Acanthamoeba* spp. by using many concentrations of insulin in different exposure times

Concentration IU/ml	Survival rate (%)			Mean of survival rate in concentrations	P Value
	5 min	10 min	15 min		
0	100	100	100	100	p>0.05
25	77.00	60.90	44.90	60.90	p<0.05
50	57.04	45.30	31.90	44.90	p<0.05
100	12.00	0.00	0.00	4.00	p<0.05
Mean of survival rate in exposure times	60.78	50.91	51.90		

Data are represented as a percentage (%). P value less than 0.05 is indicated a significant difference in compared control

Discussion

The current study showed a positive relationship between the concentration of in vitro insulin and the survival rate of *Acanthamoeba*. Levi-Schaffer and Smolarsky (1981) revealed in their study during schistosomula infection, insulin could increase glucose uptake and oxygen, resistance to antibodies, and life span.

Tokarz *et al*. (2018) noted that insulin enhances the entry of glucose into human cells, as Baig and Khaleeq (2020) revealed that insulin causes the spread and growth of the vegetative phase of

Acanthamoeba at doses between 2.98 and 5.97 micromol/ml compared to control.

It also contributed to reducing the rate of potassium ion K+, and this was observed in 12 million vegetative stages of *Acanthamoeba* growing in PYG medium when exposed to high levels of insulin. Insulin also increases Ca+, as high doses increase free calcium inside the parasite. While the current study showed a gradual lethal activity for the vegetative stage, the rates of gradual death of the parasite were based on three doses and different concentrations. Our results agree with the previous study, which attributed the increase and decrease of some ions to the weakening of the parasite's growth.

Also, our study suggests that insulin could increase autophagic digestion of cytosolic protein, which is a part of the process of autophagy32 in *Acanthamoeba* trophozoites. This suggestion agreed with Baig and Khaleeq (2020) when they studied the effect of insulin on *Acanthamoeba* spp. .

Recommendations

This study may be necessary in eliminating the parasite inside the host's body. Therefore, we recommend extensive studies to determine the lethal dose for the parasite, provided that it is safe for the host's cells.

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