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Diphtheria Antitoxin Antibodies in Type II Diabetes Mellitus with Toxin Neutralization Method

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Abstract

Objective: The study was conducted on 310 volunteer diabetes mellitus patients admitted to the outpatient clinic and 200 controls in Kirikkale University School of Medicine. Patients and controls were given standard questionnaires including demographic data and vaccination history.

Research Design and Methods: Sera samples were kept at -20 ^{0}C until used. Toxin neutralization (TN) method was applied to measure the diphtheria antibody levels in the sera samples. By the TN test < 0.01 IU/ml levels were assessed as susceptible; ≥ 0.01 - < 0.1 IU/ml levels as basic protection; and ≥ 0.1 IU/ml levels as full protection status. The statistical analysis was done by using SPSS 8.0 program.

Results: The susceptibility rates, basic protection rates and full protection rates in patient and control groups were 18.1%, 42.5%, 81.9% and 16.5%, 36.5%, 83.5%, respectively. Results were not significantly different for patient and control groups at all titer intervals (x^2=2.966, p=0.227). A tendency of difference in only older age patients was noted in the patient group although it was not statistically significant (x^2=20.923, p=0.052). No significant difference was found in the control group (x^2=15.908, p=0.196). A statistical difference was not identified between the groups in terms of mean titer, educational level, age and gender.

Conclusions: There was not a statistically significant difference between those groups. For this reason, people whose antitoxin levels were susceptible had to be determined and vaccinated.

Key words: diabetes mellitus, diphtheria antitoxin level, toxin neutralization test

Amaç: Bu araştırma Kırıkkale Üniversitesi Tıp Fakültesi hastanesine ayaktan başvuran 310 gönüllü diyabetes mellitus hastası ile 200 sağlıklı kontrol grubunda yapıldı. Hastalar ve kontrol grubuna demografik bilgileri ve aşılama hikâyesini de içeren standart anket uygulandı.

Araştırma Dizaynı ve Metot: Serum örnekleri alınarak -20 ^{0}C 'de saklandı. Toksin nötralizasyon (TN) metodu ile serum örneklerinde difteri antikor düzeyleri ölçüldü. Bu metotla, < 0.01 IU/ml düzeyler hassa; ≥ 0.01 < 0.1 IU/ml düzeyler temel koruyucu ve ≥ 0.1 IU/ml seviyeler tam koruyucu düzeyler olarak değerlendirildi. İstatistiksel analiz SPSS 8.0 programı kullanılarak yapıldı.

Sonuçlar: Hassasiyet, temel koruyucu ve tam koruyucu düzeyler hasta ve kontrol grubunda sırasıyla %18.1, %42.5, %81.9 ve %16.5, %36.5, %83.5 olarak ölçüldü. Sonuçlar tüm titre aralığında incelendiğinde hasta ve kontrol grubu arasında fark bulunmadı (x^2=2.966, p=0.227). Sadece daha yaşlı hasta bireyler arasında, istatistiksel olarak anlamlı fark olmasa da farka eğilim tespit edildi (x^2=20.923, p=0.052). Kontrol grubunda da ise aynı yaş grubunda anlamlı fark yoktu (x^2=15.908, p=0.196). İstatistiksel farklılık her iki grupta eğitim düzeyi, yaş ve cinsiyet üzerinde de bulunmadı.

Tartışma: Her iki grup arasında istatistiksel olarak anlamlı bir fark bulunmadı. Bu nedenle, antitoksin düzeyi hassas olan kişiler tespit edilip aşılanmalıdır.

Anahtar kelimeler: diyabetes mellitus, difteri antitoksin düzeyleri, toksin nötralizasyon testi

Introduction

Diphtheria poses a threat on the non immunized or inadequately immunized people due to the increase in global travels and emergence of the epidemiological strains all around the world. In the developed countries, the incidence rate is low because of effective immunization programs; however the number of susceptible persons has been increasing due to lack of repeat vaccine dose administration on a periodical basis and exposure to toxicogenic strains [1, 2].

In Turkey, diphtheria immunization is achieved via administration of 4 doses of diphtheria- tetanus-pertussis combined vaccine in the first two years of life. However, it is not a common practice to administer the repeat doses regularly after adolescence. In non-insulin depended diabetes mellitus (NIDDM) patients, the incidence of soft tissue, respiratory and urinary tract infections have increased. This increased incidence rate in a way is linked with disturbed leukocyte functions. It was shown that the phagocytosis and bactericidal activity of macrophage and polymorph nuclear leucocytes have been reduced. Besides, there is disturbance in the response of the specific immune systems such as B lymphocytes [3, 4].

Local and systemic toxicity due to *C. diphtheria* infection is caused by an extracellular toxin with antigenic properties. Immunity against diphtheria is dependent upon the specific IgG antibody (IgG-DT Ab) that develops to combat this toxin. Since neutralization tests are time-consuming and expensive, different serological procedures are applied in clinical practice.

Some of the tests employed are the passive hemagglutination test (PHA), diphtheria antitoxin-ELISA test, toxin binding ELISA (ToBI-ELISA) tests. WHO suggests toxin neutralization test (TN Vero) as reference test method since it represents the immune status better [5]. It is acceptable in practical terms to do the screening tests by ELISA and then carrying out further investigations with TN test on titers of <0.1 IU/ml [5].

In this study, our aim was to test the hypothesis that diabetics might become more sensitive to diphtheria with age. To test this hypotheses diphtheria IgG antitoxin the TN Vero test was applied as serological method in type II diabetic adult patients and in healthy control group.

Material and Methods

The study was designed in voluntary patients admitted to outpatients clinics of the Departments of Internal Medicine and Infectious Diseases and Clinical Microbiology, School of Medicine in Kirikkale, Turkey. The diagnosis of non-insulin-dependant diabetes mellitus (NIDDM) was diagnosed by elevated levels of fasting glucose in the absence of ketonemia. Patients with positive HIV test; autoimmune disease; clinically significant hematological, hepatic, metabolic, renal diseases; active systemic bacterial or fungal infection; performed organ transplantation; receiving any treatment due to malignant neoplastic disease in the last 5 years and alcohol and drug abuses were excluded from the study.

Each patient underwent a standardized questionnaire about age, sex, occupation, education, duration of illness, and location where primary and booster immunization were given, whether the patient had been fully immunized against diphtheria, the number of booster vaccinations and the number of years passed since the last vaccination.

The sera were frozen and stored at -20 °C until diphtheria antitoxin testing by using ELISA and TN was performed. Freezing and thawing of seem was kept at a minimum level.

Serological Analysis: Diphtheria antitoxin was measured by toxin neutralization.

Diphtheria toxin neutralization (TN) test: The diphtheria toxin neutralization test in *Vero* cells (green monkey renal epithelium) was used. Serial two-fold dilutions of serum were mixed with diphtheria toxin (0.0002 Lf) and incubated for l h at room temperature. Then 50 µl of suspension containing $2.5x10^5$ of *Vero* cells/ml were added to each well. The plates were gently shaken, covered with a polyethylene seal and incubated at 37^0C for 6 days. Changes in color from red to yellow indicated metabolic inhibition by the toxin due to neutralization of the toxin by antitoxin antibodies. On the basis of concurrent testing with the WHO reference serum (1.5 IU/ml, National Institute for Biological Standards and Control, Hertfordshire, UK), the antibody titer was expressed in IU/ml.

Statistical Analysis: Statistical analysis was performed by using SPSS 8.0 (Statistical Package for Social Sciences for Windows; Chicago, IL, USA). Mann Whitney *'U'* test, Kruskal Wallis, Pearson $\chi 2$ tests and Spearman's correlation linear regression analyses were used to analyze data. Statistical significance was accepted if p value was less than 0.05.

Results

Age and sex distributions of patients and healthy controls were summarized in Table 1. There were no statistical difference between groups based on these parameters (Pearson $\chi 2$ for age: 15.908, p=0.196 and for sex: 1.103, p: 0.981).

Mean antitoxin level (by TN test) was found to be 0.2647 ± 0.863 IU/ml (median 0.063) and 0.3058 ± 0.850 IU/ml (median 0.093) for patient and control groups, respectively. There was not any statistically significant difference between the groups in anti toxin levels (MWUT z=-1.735, p=0.083). Moreover, mean antitoxin levels did not demonstrate any statistically significant difference by sex (MWUT z=-1.337, p=0.181).

Total protection rate (≥ 0.01 IU/ml) was found as 81.9% for patients and as 83.5% for control cases, by using TN Test. There was not any statistical difference between patient and control groups based on preventive diphtheria titers (x^2=2.966, p=0.227) (Table 2). No statistical difference was observed in the control group (x^2=15.908, p=0.196) with respect to age. However, in the patient group, a slightly significant difference was identified although there was not a statistically insignificant difference (x^2=20.923, p=0.052). The highest antitoxin levels were found in 4, 5 and 6 decades of age (Table 2).

There was no statistical difference was between patient and control groups in diphtheria titers less than 0.1 IU/ml, obtained via TN test, (MWU z=-1.213, p=0.225). The same relation was true for basic protective and susceptible titers (respectively, MWU z=-0.066, p=0.947 and MWU z=-0.496, p=0.620).

There was not any correlation between diphtheria antitoxin levels and educational status of the patient and control groups (Pearson χ^2=6.726, p=0.242 and χ^2=7.809, p=0.167, respectively). Moreover, there was not any correlation between diphtheria antitoxin levels and the duration of the disease (r= 0.068, p= 0.233).

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Table 1: Breakdown of age and sex of the patient and control groups

Groups	**Patient**		**Control**		**Total**
	Male	Female	Male	Female	
Number (n)	104	206	72	128	510
Percentage (%)	**33.5**	**66.5**	**36**	**64**	**100**
Disease Duration	75.9±4.37 mths (1-420 mths)				
Age range	24-85		28-80		24-85
Average age	55.45±11.56		55.81±11.38		55.48 ± 11.57 yrs

Table 2: Distribution of diphtheria antitoxin levels by age in patient and control groups.

Age range (yrs)	**Antitoxin concentration (IU/ml) (TN Vero)**											
	< 0.01				**≥ 0.01 - < 0.1**				**> 0.1**			
	patient		***control***		***patient***		***control***		***patient***		***control***	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
20-29	2	**0.6**	1	**0.5**	-	**-**	1	**0.5**	1	**0.3**	-	**-**
30-39	11	**3.6**	6	**2.3**	10	**3.2**	4	**2**	8	**2.6**	7	**3.5**
40-49	11	**3.6**	3	**1.5**	21	**6.8**	15	**7.5**	27	**8.7**	21	**10.5**
50-59	14	**4.5**	12	**6**	41	**13.2**	20	**10**	37	**11.9**	31	**15.5**
60-69	13	**4.2**	9	**4.5**	49	**15.8**	27	**13.5**	33	**10.6**	23	**11.5**
70-79	4	**1.3**	2	**1**	9	**2.9**	5	**2.5**	15	**4.8**	12	**6**
80-89	1	**0.3**	-	**-**	2	**0.6**	1	**0.5**	1	**0.3**	-	**-**
Total	**56**	**18.1**	**33**	**16.5**	**132**	**42.5**	**73**	**36.5**	**122**	**39.4**	**94**	**47**

Conclusion

It is necessary to have the primary vaccination completed by two years of age, booster dose administered during schooling age, and 90% immunity rate verified by conducting appropriate serological studies to control the diphtheria epidemic [1]. Antibody level of ≥ 0.01 IU/ml reflects a basic protection level against the toxic symptoms of the disease. The basic value desired for individual protection is ≥ 0.1 IU/ml. Preventive antibody levels must be at least 90% in children and 70-75% in adults to prevent and eliminate the disease via immunity [6, 7].

All countries must conduct serological studies to assess the immunity levels against diphtheria; and for preventing the reoccurrence of diphtheria, the protective immunity is a must for life long. Because the protection level against toxin producing strains of *C.diphtheria* diminish after certain time, so far WHO suggests a booster dose for adults every ten years [1, 8]. Besides, adults coming from high risk areas, refugees, and people with immune system deficiency must be protected.

In a study conducted in the Wales and the UK, it is shown that protection against diphtheria is more that 80% between two and 20-24 years of age, however it goes down to 29% above 60 [6]. During pre-vaccination period, diphtheria epidemic had been seen in the adolescent population. Yet, in 1997 epidemic the adult population was affected more in Russia. Majority of the cases occurred in the age group above 14 (68% of the cases) and 78% of the deaths were observed in this population. The highest mortality rate was observed in the ages of 40-49 [9]

In the 90's the diphtheria epidemic in Former Soviet Republics (Newly Independent States) had been the first large scale epidemic in the industrialized world for the last three decades. From 1990 to 1994 more than 140.000 new cases and 4000 deaths were reported. The most significant factor about this epidemic was the high level of susceptibility in the adult population [7, 10, 11, 12]. Studies conducted in these states and also Western Europe and USA have clearly shown that in 20-60% of the adult population above 20 has susceptibility for diphtheria [13, 14, 15, 16]. In diabetic patients with a marked immune system failure, the weakening of the diphtheria specific immunity can sometimes be significant [3, 4]. However, studies targeting the antitoxin level determination in such patient population are very limited in number.

In this study, the protective antitoxin level was found to be 81.9% with TN (≥ 0.01 IU/ml). In the control group this was 83.5%. No statistically significant difference was found in the control group with respect to age. As the age increases the number of susceptible persons would also increase. Although not significant, this was a slightly significant difference (p=0.052). The highest protection level was documented in 4, 5 and 6th decades of age. Protection level was expected to be the highest in the young adult population but it could not be confirmed. This may be due to limited patient population.

Susceptibility for diphtheria increases by age [7]. There are some minor differences by sex in that regard. It is

partially higher in the female population. This can be explained by the immunization during military service and more frequent injuries and following Td vaccinations. Some studies report that the antibody levels are higher in female population [13, 16]. We did not find any statistically significant differences both in the patient and control groups (p=0.181). Likewise, there was not a significant correlation between the educational level and antitoxin levels (patient, p=0.242; control, p=0.167).

Studies on the seroprevalence of the vaccine preventable diseases, as well as the reevaluation and rationalization of the immunization policies against those diseases are warranted. Especially humoral and cellular immune deficiency associated conditions must be followed closely. Our results did not confirm that diabetics were more sensitive to diphtheria compared to controls. As in the normal population, the diabetic patients must be given priority for immunization because they are more prone to injuries, though immunization should be provided to all the members of the society.

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