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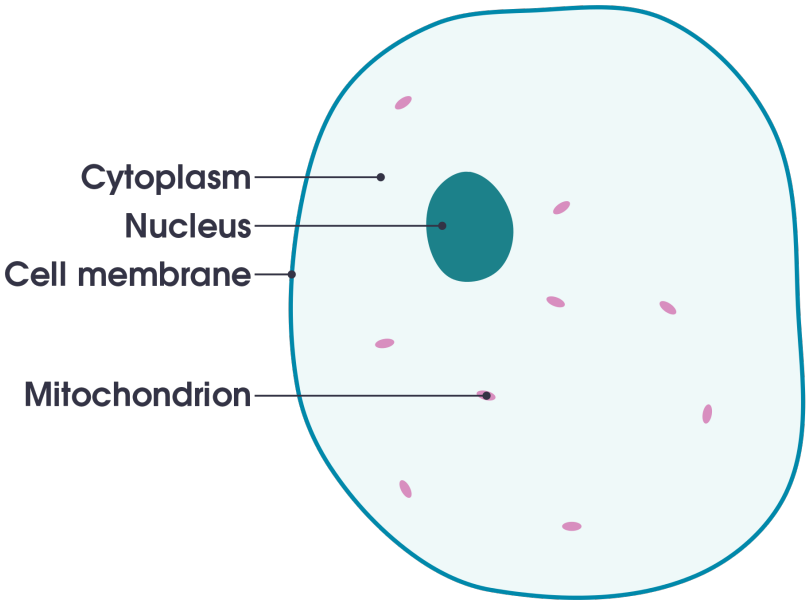

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Original Research

Prevalence of Hepatitis C virus infections among the general population of Buner, Khyber Pakhtunkhwa, Pakistan

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Abstract

Introduction: In the current study, prevalence of hepatitis C virus and its routes of transmission were determined in district Buner, Khyber Pakhtunkhwa, Pakistan. **Methods**: Total 230 blood samples for HCV were collected from 7 tehsils of District Buner. Diaspot rapid HCV strips manufactured by Diaspot diagnostic USA were used for the detection of HCV-Ab in the blood. **Results**: Out of 230 blood samples collected from patients, 158 were found positive for HCV infection. Of the 158 positive cases, 102 (64.55%) were male and 56 (35.45%) were female patients. The high rate of Hepatitis C infection was found in the age group of 41-50 years that was 68 (29.56%), followed by age group of 20-30 years that was 60 (26.08%), 31-40 years that was 54 (23.47%). The lowest infection was found in age group above 70 years. **Conclusion**: It is concluded from the study that such high proportion of hepatitis C prevalence will result in high rate of mortality and morbidity in the area. Possible steps are required for the prevention and cure of such viral infections.

Keywords

Hepatitis C Infection, Prevalence, Buner, Pakistan

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Introduction

Hepatitis C virus was first identified and reported in 1989. Consequent survey on the population found that hepatitis C was accountable for about 90% of all transfusion associated belongings of non–A, non-B hepatitis (Aach et al., 1991; Alter et al., 1989; Ikeda et al., 1994).

Hepatitis "C" virus (HCV) is a spherical, enveloped, single-stranded RNA virus belonging to *Flaviviridae* family and genus *Hepacivirus*. Hepatitis "C" virus is highly heterogeneous and is classified into 11 different genotypes of which 6 are major genotypes and are further classified into many subtypes based on the genomic sequence heterogeneity. Genotypes of HCV are the intrinsic characteristics of the infecting virus strain and do not change over time (Muhammad and Jan, 2005). Hepatitis "C" is an infectious disease affecting primarily the liver, caused by the hepatitis C virus (HCV). The infection is often asymptomatic, but chronic infection can lead to scarring of the liver and ultimately to cirrhosis, which is generally apparent after many years. In some cases, those with cirrhosis will go on to develop liver failure, liver cancer or life-threatening oesophageal and gastric varices (Funda, 2001).

Hepatitis C virus (HCV) infection is a main global health concern. According to the world health organization, the previous worldwide burden of hepatitis C included only burden from severe HCV infectivity (Organization, 2004a). Recent burden estimates shows that globally there were 54,000 deaths and 955,000 disability adjusted life-years associated with severe Hepatitis C Virus infection. Chronic sequelae infections result in Hepatitis C Virus infection (Perz et al., 2006). According to the most authentic data, three to four million develop hepatitis C each year, 170 million people develop chronic infection which leads to liver disease including cirrhosis and liver cancer, and 350,000 deaths each year are due to all HCV-related causes (Perz et al., 2006).

Hepatitis "C" Virus (HCV) is responsible for infecting about 170 million people globally and the second most common cause of viral hepatitis (Idrees and Riazuddin, 2008). About 250,000 to 350,000 deaths occur per year because of cirrhosis, end-stage liver disease, and hepatocellular carcinoma (Chevaliez and Pawlotsky, 2007) due to chronic hepatitis C infection.

Hepatitis C and Hepatitis B is responsible for morbidity and mortality and is a serious health problem globally as well as in Pakistan (Ahmad et al., 2004). In Pakistan, the prevalence of hepatitis C virus infection is higher in rural areas than urban areas (Aziz et al., 2010). As estimated by the Economic Survey of Pakistan, the government of Pakistan spends about 0.75 of GDP on health sector (Akram, 2007). On such low provision of funds, the public sector lacks quality in its services. Pakistan had just 7.3 physicians and 4.7 nursing personnel per 1000 population (Organization, 2004b).

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A study was conducted in District Mardan, Khyber Pakhtunkhwa (KPK) province of Pakistan to determine the prevalence of hepatitis C virus infection. They report the overall prevalence of HCV in Mardan as 3.66%. They report the high burden of HCV in age group of 41-50 that was 7.4%. The disease burden was higher in male population that was 4.5% as compared to female population that was 2% (Arshad, 2012).

A detailed study was conducted in the MMT Hospital, D I Khan and Ibrahimi Hospital and Trust, Peshawar. They reported the highest prevalence of HCV in males that was 282 (53.1%) and females 255 (46.9%). The HCV genotype details were shown as: Type 3a were 355 (66.11%), 3b were 14 (2.61%), 2a were 40 (7.45%), 2b were 5 (0.93%), type 1a were 9 (1.67%), 1b were 9 (1.67%) and patients with mixed types 2a and 3b, 3 (0.56%) were combined 3a and 3b and unable to type were 101 (18.81%) (Khan et al., 2014).

The earth quake in October 2005 in Hazara Division took more than 100,000 lives and most of others were seriously injured (Ahmad, 2005; Sheikh, 2005). In such situation, it is feared that the transfusion of unscreened blood might have increased the risk of hepatitis C transmission (Khan et al., 2003). But the fact remains that there is no accurate information available about the prevalence of hepatitis C in the area. A population based survey was conducted to estimate the prevalence of hepatitis C in the area. The accurate information regarding the prevalence of hepatitis C in the area is required to measure the shock of any interference in the area (Shepard et al., 2005).

In Pakistan, the major ways of hepatitis C virus transmission are the contaminated instruments use in medical practice, unscreened blood and blood product transfusion, the uses of intravenous drugs, barber shops, piercing of ear and nose, unhygienic habits and poor medical practice by non-qualified people (Ali et al., 2009a; Raja and Janjua, 2008; Zuberi et al., 2008). Among them, one of the major transmission route is lack of proper screening of the transfusing blood (Luby et al., 1997).

According to World Health Organization, about 80% people infected with hepatitis C have no signs and symptoms (Organization, 2011). The symptoms reported in 20-30% of people recently infected with hepatitis C are tiredness, abdominal pain, poor appetite, or jaundice (Prevention, 2012).

The result of antibodies to various viral antigens shows infection with the virus and in most cases portrays a constant infection (Mc Lean, 1997). The duration of hepatitis C might be extended and dangerous and the patients with hepatitis C will develop no sign and symptoms for many years after the inception of infection (Mc Lean, 1997).

Majority of the studies conducted are based on hospitalised patient data due to which true prevalence of HCV infection could not be determined. So far, no population based study has been conducted to investigate the prevalence of

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active HCV infection or anti-HCV antibodies in district Buner. This study aims to determine the prevalence of anti-HCV antibodies and HCV infection in the general population of district Buner.

Methods

Introduction to District Buner

District Buner is situated between 34^0-11′ and 34^0-43′ North – Latitude and 72^0-13′ and 72^0- 45′ East – Longitude in Khyber Pakhtunkhwa province of Pakistan. The District is surrounded by Swat district on North, Malakand Agency on the West Mardan district on the South and Hazara Division on the east having altitudinal range 366-291 m, with a total area of 1865 km^2 and population of 506,048 individuals (Khan et al., 2012) (**Fig. 1**).

District is subdivided into 7 tehsils, namely

1. Tehsil Daggar.
2. Tehsil Gagra.
3. Tehsil Gadezai.
4. Tehsil Mandar.
5. Tehsil Chagharzi.
6. Tehsil Amazi.
7. Tehsil Khodo Khel.

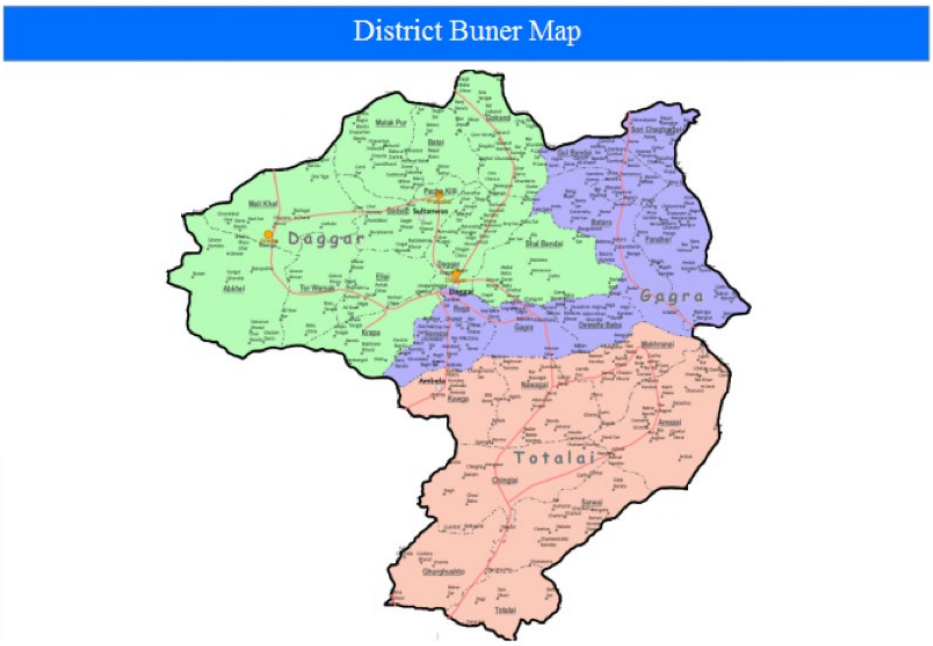

Figure 1. Map of District Buner.

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Samples collection

During this study, two hundred and thirty blood samples were collected from the seven tehsils of district Buner, Khyber Pakhtunkhwa, Pakistan. The collection was done through specialised proforma which will contain the whole information about the patient. After completing the initial data, 3 mL blood was collected through (Shifa) disposable syringes. The collected blood was then transferred to serum tubes (ATLAS-LABOVAC Italiano) containing oxalate to minimise the chance of blood clotting. The blood was then transferred to the Human Genetics Lab of Hazara University, Mansehra. The blood was then centrifuged to separate serum. The separated serum was then transferred to Eppendorf tubes and was stored at -80°C.

Assay for detection of HCV Antibody

The strips used for the detection of HBsAg in the blood were DiaSpot® HCV-Ab Test strips (manufactured by DiaSpot Diagnostics, USA), Global® HCV-Ab Kit (manufactured by Global Diagnostics, USA). These methods are immunochromatographic and qualitative in nature and identify the presence of HBsAg in human blood and might interpret in vitro having more than 99.9% sensitivity and 98.6% specificity. The manufacturer's specifications were followed to perform the test.

Approval from ethical community

The research was approved from the ethical community of district Buner.

Results

A descriptive study was conducted to find out the prevalence of HCV infection in the general population of district Buner, Khyber Pakhtunkhwa, Pakistan. In this study, 230 blood samples were collected from the whole district Buner along with the proforma for taking whole information from the patient. Among the total 230 blood samples, 139 were males and 91 were females. The data was collected from different age groups. The infection rate was different in different age groups. The details are given in **Table 1** and **Figure 2**.

Signs and symptoms

During the study, patients were interviewed about the HCV. The signs and symptoms of the patients were recorded on the designed proforma. The patients were showing different signs and symptoms. Fatigue was common in all patients that was 100%, joint pain was also common and was 100%, redness on the palm of hands was 100%, damage to brain and nervous system was 90.50%, belly pain was 75.94%, sore muscles was 74.68%, jaundice was 72.78%, itchy skin was 56.96%, shrinking of muscles was 29.11%, cluster of blood vessels

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below skin was 23.41%, swelling of belly, legs, feet was 14.56%, dark urine was 4.43%, bleeding from enlarged veins in digestive tract was 0%. The details of signs and symptoms of HCV are given in **Table 2**.

Table 1. Table showing sex and age wise distribution of data

Age	Male (%)	Female (%)
20- 30 years	34	26
31- 40 years	38	16
41- 50 years	36	32
51- 60 years	27	17
61- 70 years	3	---
Above 70 years	1	---
Total	**139**	**91**

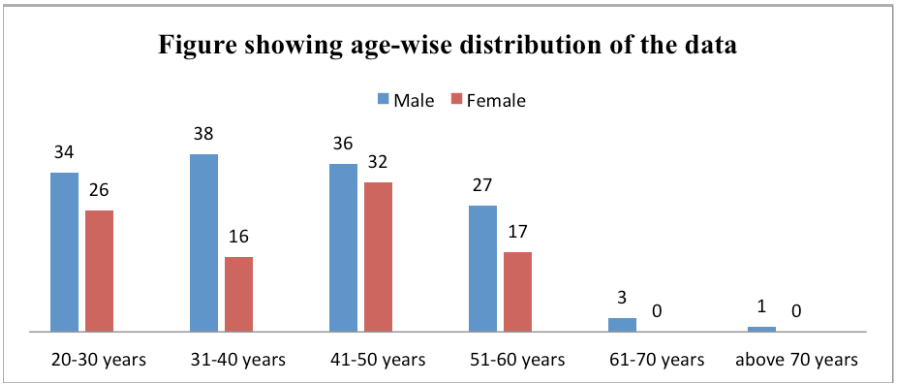

Figure 2. Figure showing age-wise distribution of the data.

Routes of transmission

In this study, the routes of transmission of hepatitis C were determined. The major ones contributing for hepatitis C in District Buner were Intravenous Drug Users (IDUs), Blood transfusion (BT), surgery, dialysis, GYN wards, barber shops, piercing in unclean environment and unsafe sex.

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Table 2. Patients presenting signs and symptoms of HCV

Signs and Symptoms	Frequency	Percentage
Fatigue	158	100%
Joint pain	158	100%
Belly pain	120	75.94%
Itchy skin	90	56.96%
Sore muscles	118	74.68%
Dark urine	7	4.43%
Jaundice	115	72.78%
Redness on the palm of hands	158	100%
Clusters of blood vessels just below the skin (Chest, Shoulders, and Face)	37	23.41%
Swelling of your belly, legs, and feet	23	14.56%
Shrinking of the muscles	46	29.11%
Bleeding from enlarged veins in digestive tract	--	--
Damage to brain and nervous system	143	90.50%

Discussion

Hepatitis is a public health problem worldwide. Infectious diseases including Hepatitis C are major health issues in various developing countries including Pakistan (Ali et al., 2009a; Khan and Siddiqui, 2007). According to an estimate of the World Health Organization, hepatitis C has effected almost 3% population of the world, an estimate of 170 million people, with an increase in three to four million new cases every year (Raza et al., 2007).

In current study, we attempt to investigate the prevalence of hepatitis C virus infection and its modes of transmission in district Buner, Khyber Pakhtunkhwa, Pakistan. In current study, we collected 230 samples from whole Buner district in which 158 were positive and 72 were found negative for HCV infection.

A study conducted by Huda *et al* (2013) showed that the infection rate of hepatitis C virus was high in males that was 20% while in females it was 13.33% (ul Huda et al., 2013). A study conducted by Arshad *et al* (2012) reported high prevalence of hepatitis C in male population that was 4.5% compared to females

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that was 2% (Arshad, 2012). According to Ilyas *et al* (2011), the rate of HCV infection was higher in males as compared to females that was 2.60% and 1.68% respectively (Ilyas et al., 2011). Other studies conducted in Pakistan also shows the higher frequency of Hepatitis C in males as compared to females (Khan and Siddiqui, 2007; Khan et al., 2003; Mashud, 2004). The reason behind high prevalence of Hepatitis C infection in males might be freedom and social mobility in which there is high risk of contracting viral infection. In our study, the total number of 230 samples were collected in which 158 samples were positive. In these 158 samples, 102 were males and 56 were females. Our result is supported by the previous results in which the rate of incidence of hepatitis C is higher in males than that of females.

According to Ahmad *et al* (2004), hepatitis C and hepatitis B have high prevalence in age group of 55-64 years and low in the age group of 25-34 years (Ahmad et al., 2004). Arshad *et al* (2012) reported highest prevalence of HCV in age group 41-50 that was 7.4%, followed by age group 51 and above that was 6.36% (Arshad, 2012). Different age groups and many other factors are associated with prevalence of Hepatitis C virus infection in different regions of Pakistan (Khan et al., 2011). In our study, the high rate of Hepatitis C infection was found in the age group of 41-50 years that was 68 (29.56%), followed by age group of 20-30 years that was 60 (26.08), 31-40 years that was 54 (23.47%) while the lowest infection was found in age group of above 70 years that was 1 (0.43).

According to Adeyemi *et al* (2014), blood transfusion is one of the major route of viral hepatitis. The false negative results in the blood banking results in the transfusion of infected blood to an uninfected person (Adeyemi et al., 2014). There is an important role of blood transfusion in the epidemiology of HCV infection (Bialek and Terrault, 2006). Study on blood donors in Kathmandu, Nepal results in 0.64% seroprevalence of HCV (Shrestha et al., 2009). Imoru *et al* (2003) reported 0.4 % prevalence of hepatitis C virus antibody in Kano State (Imoru et al., 2003). The unscreened blood products are one of the leading causes of hepatitis C virus transmission. The incidence of post-transfusion of hepatitis C in United States has dropped from 3.84% to 0.57% after the introduction of HCV screening in 1990 (Donahue et al., 1992). In our study, blood transfusion was one of the route for hepatitis C virus infection. In previous literatures, the prevalence of HCV in the blood donors has showed lower, but we cannot neglect the risk of transmission of HCV through blood and its products.

Contaminated surgical instruments, contaminated needles and syringes are one of the important source of HCV transmission globally (Hauri et al., 2004; Luby et al., 1997). Developing countries have the higher risk of incidence of HCV than developed countries (Bialek and Terrault, 2006). Egypt has the highest rate of hepatitis C incidence that is five-fold than in developed countries (Strickland, 2006). HCV can transmit from infected patients to health workers through needle injuries with the infection rate about 1.8% (Beltrami et al., 2000). The higher rate of infection had been reported in the dialysis patients. Saudi Arabia had showed

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the high rate of prevalence in the haemodialysis patients that is 9.24% as that of blood donors that is 0.30% (Qadi et al., 2004). These studies show the transmission of hepatitis C through haemodialysis patients due to poor sterile techniques, poor cleaning of dialysis machine and other possible ways increase the risk of hepatitis C transmission among the patients (Zampieron et al., 2004).

Intravenous drug usage is the prominent mode of transmission of hepatitis C virus in the developed countries. In USA, the incidence of hepatitis C cases increased from 31% in 1994 to 38% in 1999 and reached to 45% in 2003 (Prevention, 2005).

Among the 310 drug users in Antwerp, prevalence of anti-HCV antibody was 71% and in Limburg in Belgium was 46% (Matheï et al., 2005). The Hepatitis C European Network for C-operative Research (HENCORE) group had reported the prevalence of 80% hepatitis C among intravenous drug users (IVDU) (Touzet et al., 2000). There was 36.6% positivity of hepatitis C in the intravenous drug users in Sydney, Australia and 74% hepatitis C positivity in the intravenous drug users in Melbourne, Australia (Bradshaw et al., 2005; Maher et al., 2004). In the recent study in London, England, the 428 intravenous drug users were under the age of 30 having 44% antibodies to hepatitis C compared to 4% with HIV, this is the incidence of 41.8 cases per 100 persons (Judd et al., 2004). Most of such patients are not aware from the risk of transmission and sometimes they do not know that they are infected, therefore screening of such group is important.

In a study, conducted by Ali *et al* (2009b) the major symptoms of hepatitis C virus were high bilirubin with high liver enzymes that was 17.94%, yellowish eyes with skin colouration was 15.39% and dark yellow urine with diarrhoea was 10.25%, fever was 15.38%, vomiting was 12.82%, loss of appetite was 7.69%, stomach pain was 5.12% and tiredness was 2.56%. They also reported that 56.41% patients were infected with HCV, but were presenting no symptoms (Ali et al., 2009b). During our study, the patients were showing different signs and symptoms. Fatigue was common in all patients that was 100%, joint pain was also common and was 100%, redness of the palm of hands was 100%, damage to brain and nervous system was 90.50%, belly pain was 75.94%, sore muscles was 74.68%, jaundice was 72.78%, itchy skin was 56.96%, shrinking of muscles was 29.11%, cluster of blood vessels below skin was 23.41%, swelling of belly, legs, feet was 14.56%, dark urine was 4.43%, bleeding from enlarged veins in digestive tract was 0%.

Approximately, 30% of health care workers having hepatitis C infection was reported from one of the teaching hospital at Abbottabad, Pakistan (Sarwar et al., 2007). Along with this, the unclean dental equipment is common in almost every dental clinic of Pakistan (Ali et al., 2009a). In our study, it was found that most of the people infected with hepatitis C were health care workers because they have a higher risk of getting hepatitis C virus through contact with the blood and blood products of the patients.

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One of the most common factors associated with the transmission of hepatitis C is the disposal of hospital waste (Riaz et al., 2012). There are hundreds of garbage collectors of different age groups throughout the country who are associated with main recycling businessmen who deal unsterile syringes. The young scavenger in Karachi, Pakistan of about 18 to 20 years of age sells 20 to 25 syringes per day to the health care waste dealers and with these needles the same child gets injured around none to three times per week (Mujeeb et al., 2003). In our study, it was found that most of the children between the age of 12 to 18 years regularly collect the waste like syringes and other plastic materials from the hospitals and other health care providers and sell them to scrape dealers for money. During this collection, some of the needles pierce into their fingers and feet and injure them. During these injuries, there is a higher chance of developing diseases such as hepatitis C.

Hepatitis C virus (HCV) infection has become one of the most important public health concern round the globe (Costa et al., 2009). Pakistan is also facing a huge burden of these diseases. The prevalence among general public of HBV and HCV infection in Pakistan is 10% (Malik et al., 1996; Yousaf et al., 1996) and 4–10% respectively (Malik et al., 1996; Umar et al., 2000). In our study, it was found that hepatitis C is the major health problem in district Buner. Data from the whole district was collected which shows that hepatitis C is prevalent in district Buner and if the protective measures are not adopted, then it is possible that hepatitis C will become one of the major health problem in district Buner, Khyber Pakhtunkhwa, Pakistan.

Conclusion

Hepatitis C is widely prevalent in district Buner, Khyber Pakhtunkhwa, Pakistan. Such high proportion of hepatitis C prevalence will result in the high rate of mortality and morbidity in the area; therefore, possible steps are required for the prevention and cure of such viral infections. It is concluded that for the development of appropriate vaccines, research at the molecular level is required to understand the predominant genotypes circulating in district Buner, Khyber Pakhtunkhwa, Pakistan.

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List of Abbreviations

HCV: Hepatitis C Virus
HENCORE: Hepatitis C European Network for C-operative Research
IVDU: Intravenous Drug Users
ml: Milliliter
USA: United State of America
WHO: World Health Origination
KPK: Khyber Pakhtunkhwa
KM: Kilometer

Ethical approval

The research was approved from the ethical community of district Buner.

Authors' contributions

NA carried out this prevalence study. He collected the samples and got the consent of subjects. HA, KM and SS mainly supervised this study. NA, KM and KS prepared the manuscript. All authors read and approved the final manuscript.

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Original Research

Xenotransplantation of human umbilical cord derived stem cells for treatment of type 1 diabetes mellitus in mice

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Abstract

Introduction: Type 1 diabetes mellitus disease (T1D) is an autoimmune disease in which pancreatic islets are attacked by the host's immune system. Although this disease can be treated using some of the current methods, resistance to therapy can develop over time after a long usage of the treatments. Therefore, new strategies to treat T1D have been suggested. This study aims to treat T1D using a new approach to target this autoimmune disease; the approach involves the use of mesenchymal stem cells (MSCs) to induce immune modulation. **Methods**: Umbilical cord derived MSCs (UC-MSCs) were evaluated in this study. The cells were confirmed to be MSCs by surface profile markers and by *in vitro* differentiation potential into osteoblasts, adipocytes and chondroblasts. The MSCs were evaluated in a Type 1 diabetic mouse model (induced by streptozotocin (STZ)); MSCs were xenografted at a dose of 2.10^6 cells per mouse in 100 uL of saline. T1D mice injected with saline were used as placebo. Mice were monitored for body weight, blood glucose, blood insulin, glucose tolerance test and pancreas histological analysis. **Results**: Results showed that UC-MSC xenotransplantation could improve diabetes in mice. Mouse body weight significantly increased after 6 weeks of treatment. Blood glucose levels markedly decreased while blood insulin levels strongly increased towards normal range. Recovery of the insulin positive Langerhans cells was confirmed by histological analysis. **Conclusion**: Overall, our findings suggest that UC-MSC transplantation is a promising therapy for T1D treatment.

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Immune modulation; Mesenchymal stem cells; Modulatist; Stem cell therapy; Type 1 diabetes mellitus

Introduction

Diabetes mellitus is the common disease in the world. It was known as an endocrine disorder characterized by inadequate production or use of insulin, resulting in abnormally high blood glucose levels. High levels of blood glucose lead to the formation of reactive advanced glycation end-products that cause multiple complications, including blindness, kidney failure, cardiovascular disease, stroke, neuropathy and vascular dysfunction (Nathan, 1993; Tripathi and Srivastava, 2006). There are two main kinds of diabetes mellitus: diabetes type 1 and type 2. Type 1 diabetes mellitus is regarded as an autoimmune disease in which pancreatic beta cells are destroyed by host immune cells, whereas type 2 diabetes mellitus results from insulin resistance and impaired glucose tolerance (Association, 2006).

Nowadays, although diabetes mellitus type 1 can be treated by supplement of insulin, complications can arise from insulin injection and insulin resistance, which can reduce the benefits of the treatments. To overcome pancreas degeneration associated with diabetes mellitus type 1, there have been several approached aimed at promoting pancreatic regeneration. The first approach is pancreas/islet transplantation (Froud et al., 2005; Shapiro et al., 2000). Islet transplantation therapy is thought to provide good patient outcome. However, few islets are available for transplantation (Rother and Harlan, 2004); pooled islets isolated from two pancreases are typically just enough to treat a single patient.

Recently, stem cell therapies have been investigated for treatment of diabetes. Using hematopoietic stem cell transplantation (HSCT), type 1 diabetes mellitus was shown to be effectively treated (Burt et al., 2002; Voltarelli et al., 2007). HSCT provides a new immune system to replace the impaired one (Burt et al., 2002). Although HSCT has been reported as a promising therapy for T1D, there have been limitations to this method. The main challenges include lack of HSC samples that matching the patient's human leukocyte antigens (HLA) and lack of sources of HSCs (Chhabra and Brayman, 2013).

In this study, we examine a new approach in T1D treatment, based on immune modulation mediated by mesenchymal stem cells (MSCs). Immune modulation refers to the capacity of MSCs to selectively impact (namely suppress) the

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immune system. Immune cells that are the targets of MSC-mediated immune suppression include lymphocytes, dendritic cells; immune cells activated by MSCs include T-regulatory cells (Aggarwal and Pittenger, 2005; Rasmusson, 2006). Since immunomodulation by MSCs was discovered, more than 10 years ago, MSCs have increasingly been studied in the clinic for immune related diseases, especially in graft-versus-host disease (GVHD) (Bartholomew et al., 2002).

Indeed, the first stem cells (Prochymal), based on bone marrow (BM) derived allogeneic MSCs, were approved in 2012 in Canada. Since then, several other countries have begun evaluating Prochymal to manage GVHD (Chen et al., 2014; Kurtzberg et al., 2014). Recently, another form of MSCs was approved in Japan for GVHD (Konishi et al., 2016). The potency of MSCs is related to mechanisms such as contact reaction and mainly production of cytokines, including hepatic growth factor (HGF), transforming growth factor-beta (TGF-b), interleukin (IL)-10, prostaglandin E2 (PGE2), and human leukocyte antigen (HLA)-G5. In this study, human umbilical cord tissue derived mesenchymal stem cells (hUC-MSCs) were investigated as treatment for T1D in mice induced by STZ.

Methods

Cell culture

Human umbilical cord mesenchymal stem cells (MSCs) were isolated from human umbilical cord tissues according to a protocol from our laboratory (Pham et al., 2016; Van Pham et al., 2016). Briefly, the umbilical cords were collected from the local hospitals with provision of consent forms. They were transferred to the laboratory for isolation of UC-MSCs. The cord tissue was dissected into small fragments with Wharton's jelly. They were then washed twice with PBS and placed into a T-25 flask with 1 mL of culture medium (MSCCult Medium, Regenmed Lab). The flasks were incubated at 37°C for 7 days. After appearance of cells and migration from the fragments, fresh medium was replenished every 3 days until a confluency of 70-80% was reached. The cell samples were sub-cultured to the 5^{th} passage prior to use in experiments.

Immunophenotyping by flow cytometry

Cells were washed twice in PBS containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Cells were stained with anti-CD14-FITC, anti- CD34-FITC, anti- CD44-PE, anti-CD45-FITC, anti- CD73-FITC, anti-CD90- PE, or anti-HLA-DR-FITC antibody (all antibodies were purchased from BD Biosciences, San Jose, CA). Stained cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences). Isotype controls were used in all analyses.

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In vitro differentiation

For differentiation into adipogenic cells, the cells were plated and cultured for 21 days in DMEM/F12 medium containing 0.5 mM/L 3-isobutyl- 1-methyl-xanthine, 1 nM dexamethasone, 0.1 mM indomethacin, and 10% FBS (all purchased from Sigma-Aldrich). Adipogenic differentiation was evaluated by observing, via microscopy, lipid droplets in cells and following staining with Oil Red. For differentiation into osteogenic cells, cells were plated and cultured for 21 days in DMEM/F12 medium containing 10% FBS, 10^{-7} M dexamethasone, 50 μM ascorbic acid-2 phosphate, and 10 mM β-glycerol phosphate (all purchased from Sigma-Aldrich). Osteogenic differentiation was confirmed by Alizarin red staining.

Type 1 diabetic mouse model induced by streptozotocin (STZ)

Mice aged 6-8 weeks old and weighing 25-30 g were used in the study. All procedures and manipulations were performed in accordance with instructions and approval of the Animal Care Committee. The mice were housed in 12 h light /12 h dark cycle and nourished accordingly. The mice were fasted for 6 hours before STZ injection. STZ was first dissolved in cooled 0.01 M citrate buffer at pH 4.5. Type 1 diabetes in mice was induced by single intraperitoneal injection of STZ (Santa Cruz Biotechnology, Dallas, TX), at a concentration of 100 mg/kg. Mouse blood glucose was examined by One Touch Ultra (Lifescan/ Johnson & Johnson, New Brunswick, NJ). Onset of diabetes was defined as when glucose levels stably surpassed 300 mg/dL in 3 weeks.

UC-MSC transplantation

At day 21 after STZ injection, the mice which were confirmed to have diabetes were intravenously given $2x10^6$ UC-MSCs in 200 μL volume in sterile PBS. The treated mice were monitored for change in weight, blood glucose level, and serum insulin, as well as by glucose tolerance testing and evaluation of the structure of pancreatic islets.

Glucose and insulin tolerance tests

The mice were fasted 12 h before the glucose tolerance test. D-glucose was intraperitoneally injected to mice (2g/kg body weight); and blood glucose levels were measured at 0, 30, 60, and 120 minutes. The insulin was dissolved in PBS at a dose of 0.75 mg/kg. The mice were measured for blood glucose 30 minutes before insulin injection; and at 0, 30, 60, and 120 minutes after insulin treatment.

Serum insulin measurement

At the day 28, mouse blood was obtained from facial vein and the serum was collected by centrifugation at 3000 rpm in 10 minutes at 4°C. The transparent faint yellow serum was assessed for concentration of insulin by Mercodia Ultrasensitive Mouse Insulin ELISA (Uppsala, Sweden). The calibrator curve was

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constructed and the insulin concentrations from the samples were interpolated from the standard curve (Prism 6, GraphPad Software, San Diego).

Histological analysis of pancreatic islet

The harvested mouse pancreases were fixed in formalin and embedded in paraffin blocks. The 10 micrometer-thin sections were stained with Hematoxylin & Eosin following the standard established procedure. The islet structure was observed under microscopy and images were analyzed by Axio Vision Microscopy Software (Carl-Zeiss, Germany). For immunofluorescence staining, the mouse pancreases were fixed in 4% paraformaldehyde, treated with sucrose, and embedded in optimal cutting temperature compound (OCT). The 10 micrometer samples were dipped in 5% bovine serum albumin overnight at 4°C. The blocked slides were incubated with insulin antibody (Goat anti-Rabbit Alexa Fluor 488, Sigma) overnight at 4°C, then stained with FITC-conjugated secondary antibody (sc-2012, Santa Cruz Biotechnology) for 2 hours. Finally, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology) for nucleus staining and observed by fluorescence microscopy (Cell Observer, Carl-Zeiss, Germany).

Data analysis

The data were analyzed for statistical significance using GraphPad Prism software. Data were presented as mean ± SEM. When applicable, a Student's unpaired t-test and one-way ANOVA were used to determine significance; $p<0.05$ was considered to be statistically significant.

Results

Successful isolation and characterization of umbilical cord derived mesenchymal stem cells

Umbilical cord tissues were cultured according to a protocol established by us (Van Pham et al., 2016). After 5-7 days, the MSC candidates emigrated from the tissues and exhibited a fibroblast-like shape. The cells rapidly proliferated from day 10 onward and reached confluence at day 15. The cells were sub-cultured to the 5^{th} passage to produce a homogenous population. These cells were characterized for expression of MSC surface markers. Flow cytometry analysis showed that UC-MSC candidates strongly expressed CD44, CD73 and CD90, but did not express CD14, CD34, CD45 and HLA-DR. The cells also successfully differentiated into three kinds of mesoderm derived cells: osteoblasts, chondrocytes and adipocytes. During differentiation into osteoblasts, the UCMSCs changed their shape, accumulated calcium (Ca^{+2}) and magnesium

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(Mg^{2+}), and stained positive with alizarin red. The cells also successfully differentiated into chondrocytes, staining positive with Safranin O. They also differentiated into adipocytes, staining positive for Oil Red. These results were similar to the previous publication (Pham et al., 2016; Van Pham et al., 2016).

UC-MSCs significantly improve diabetes in a Type 1 diabetes mellitus mouse model

Weight of mice after transplantation

Mice treated with UC-MSC transplantation gradually increased their body weight from 28±6.77 g/mouse at day 0 (before treatment) to 31.07± 8.63 g/mouse at day 28 (post treatment) and 41.95±2.07 g/mouse at day 56 (post treatment) (**Fig. 1**). This means that the body weight of treated mice significantly increased after 2 months of treatment, compared to before treatment (41.95±2.07 g/mouse vs 28±6.77 g/mouse, respectively). Conversely, in the placebo group where mice were injected with saline, their body weight gradually decreased from 26.28±0.89 g/mouse at day 0 (before treatment) to 23.83±1.23 g/mouse at day 28 (post treatment) and to 22.3±0.52 g/mouse at day 56 (post treatment). Taken together, these results demonstrate that there was a clear effect of UCMSC transplantation on body weight recovery. After 2 months, treated mice recovered their body weight while placebo mice reduced their body weight (**Fig. 1**).

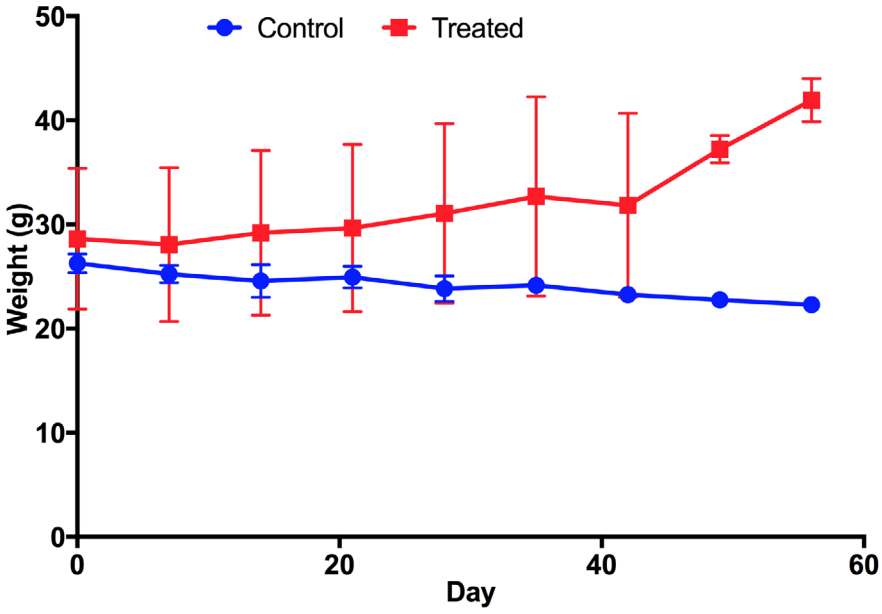

Figure 1. Changes in mouse body weight of treated mice versus placebo mice after stem cell transplantation. Mice treated with stem cells showed a gradual increase in body weight after 2 months (red line), while mice treated with placebo showed a gradual decrease in body weight (blue line).

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Blood glucose levels of mice after transplantation

The blood glucose in both treatment and placebo groups underwent significant changes during the experiment. In the treatment group, the blood glucose levels strongly decreased after 21 days of treatment - from 399.17 ± 79.24 mg/dL at day 0 (before treatment) to 334.83 ± 105.43 mg/dL at day 21 (post treatment). However, at day 28, there was a slight increase of blood glucose, from 334.83 ± 105.43 mg/dL to 343.83 ± 118.07 mg/dL; this increase was not statistically significantly ($p>0.05$). The level of blood glucose was maintained from day 21 to day 42, then remarkably decreased at day 49 (to 255.5 ±80.37 mg/dL). The achievement in reduction of blood glucose levels in diabetic mice suggested that the diabetic mice were recovering. However, we did see a slight increase of blood glucose at day 56 (314.75 ±138.71 mg/dL) when compared to day 49 (255.5 ±80.37 mg/dL), but non-significant ($p>0.05$). In the placebo group, the blood glucose levels not only decreased but also gradually increased- from 370.33±57.78 mg/dL at day 0 (before treatment) to 517.67 ±52.73 mg/dL at day 56 (post treatment) (**Fig. 2**).

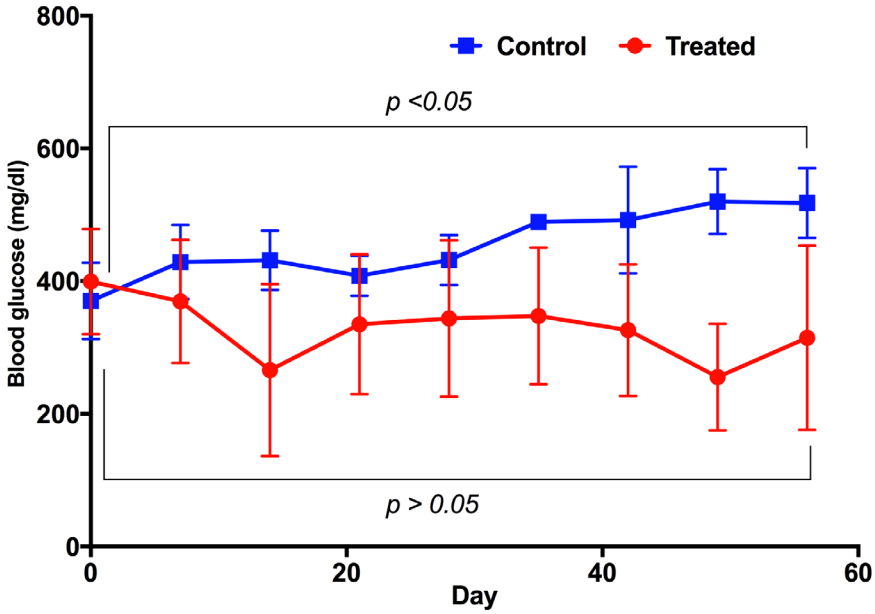

Figure 2. Changes in blood glucose levels of treated mice versus placebo mice after stem cell transplantation. UC-MSC transplantation reduced blood glucose levels of treated mice (red line). Blood glucose levels gradually increased in the placebo mice injected with saline (blue line).

Glucose tolerance test

After 28 days of treatment, mice from the treatment and placebo groups were subjected to glucose tolerance testing to determine the glucose absorbance of mice; normal (i.e. untreated) mice were used as positive controls. **Figure 3**

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shows that the responses of mice of each of the groups were different. Untreated mice were able to take in glucose after 1h, then glucose levels dropped to normal levels, similar to the level before glucose injection (**Fig. 3**; black line). In the placebo group, although the glucose level decreased after 60 mins, this level was maintained for 120 mins (**Fig. 3**; blue line). This means that absorbance of glucose in the placebo group was low. Unlike the placebo mice, the UC-MSC treated mice showed strong intake of glucose at 120 mins after glucose injection. The glucose level then returned back to the level before glucose injection (**Fig. 3**; red line).

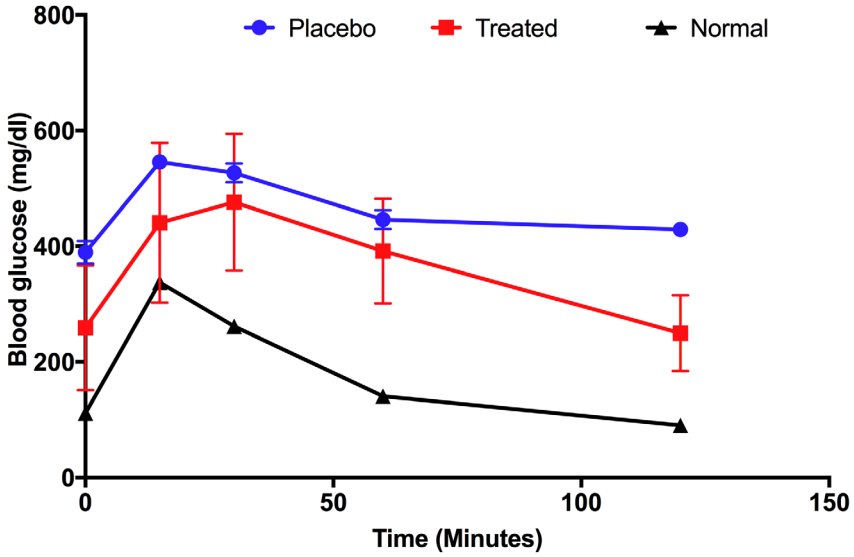

Figure 3. Glucose tolerance tests for mice in the UC-MSC treated, untreated (placebo) groups. Normal mice showed strong glucose uptake at 2 h after injection (black line). Placebo mice showed weak glucose uptake; after 1hr mice could not absorb any more glucose (blue line). UC-MSC treated mice showed good glucose absorbance (red line).

Murine insulin in peripheral blood

The level of insulin that exists in the peripheral blood reflects the recovery of the pancreas after treatment. At day 28 after treatment, peripheral blood was collected to determine the concentration of insulin. The results showed that the insulin concentration of treated mice significantly increased compared to placebo mice (1.139 ± 0.050 ug/L vs 0.30 ± 0.084 ug/L, respectively; $p<0.05$) (**Fig. 4**). More importantly, the insulin concentration of treated mice at day 28 post treatment was not significant differently than normal mice (1.139 ± 0.050 ug/L vs 1.118 ± 0.095 ug/L, respectively, $p>0.05$) (**Fig. 4**). These results show that UC-MSC transplantation helped diabetic mice recover function of their pancreas.

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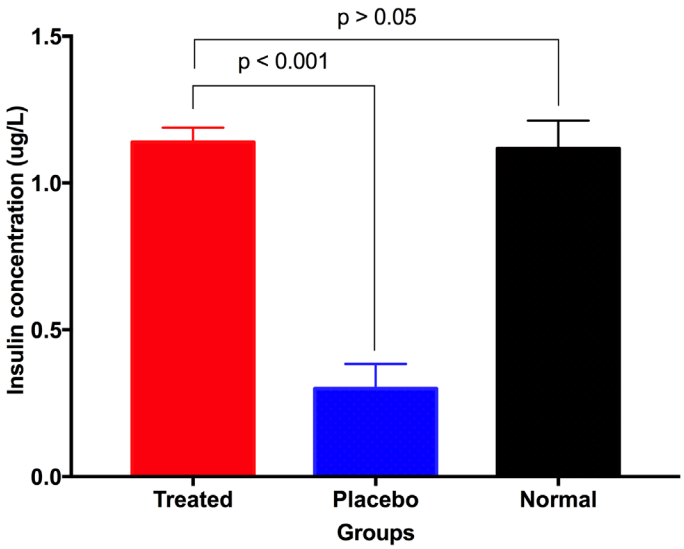

Figure 4. Insulin concentration in peripheral blood of mice in treated, untreated and placebo groups. The insulin concentration of treated mice (red column) significantly increased compared to placebo group (blue column) at 28 days post treatment. The treated mice had similar insulin concentration to normal (untreated) mice (black column).

Recovery of murine pancreatic islets after UC-MSC transplantation

The regeneration of pancreatic islets were evaluated by Hematoxylin & Eosin (H&E) staining and anti-insulin antibody staining of pancreas. H&E staining showed that STZ treated mice had injured islets and presence of pleomorphic cells (green arrow), necrotic cells (red arrow) and infiltrated lymphocytes (yellow arrow) (**Fig. 5**). The regeneration of Langerhans islets could be observed in the pancreas of treated mice and were similar to normal mice (**Fig. 5**).

The anti-insulin antibody staining showed that insulin positive cells disappeared in placebo mice, while the signal of insulin was very clear in islets of normal (untreated) mice. The injured pancreases were regenerated after UC-MSC transplantation at 28 days post treatment. Indeed, at 28 days post transplantation, insulin positive cells appeared in the pancreas of treated mice but not in the pancreas of placebo mice (**Fig. 5**). Overall, these results demonstrate that islets gradually recovered in the pancreases of UC-MSC treated mice.

Discussion

Type 1 diabetes mellitus accounts for about 5% of diabetic patients. Type 1 diabetes mellitus has been shown to be an autoimmune disease in which the host's immune system attacks the host pancreas, reducing the Langerhans islets

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as well as beta cells (Eisenbarth, 2005; Notkins and Lernmark, 2001). Insulin injection is considered as an effective method to treat T1D. However, with this method, patients must be supplied insulin for the entire duration of their life. Stem cell transplantation can rescue the pancreas from autoimmune disease by two strategies: replacement of the immune system with HSCT or immune modulation that selectively suppresses the immune system. In this study, we successfully modulated the immune system by MSC transplantation to treat T1D in a mouse model.

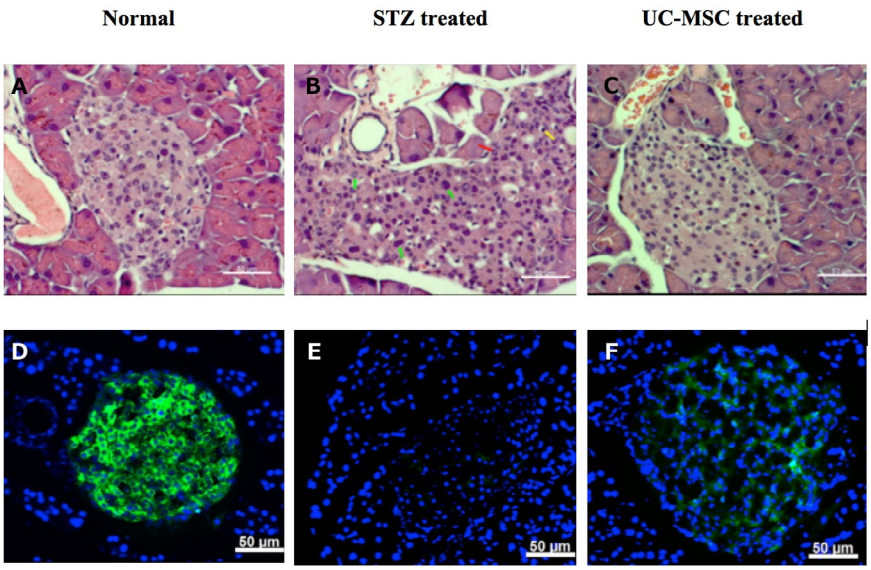

Figure 5. Histology of mouse interlobular islets. Pancreatic islets stained with Hematoxylin & Eosin; (A) The homo-morphologic pale purple islet cells in normal pancreas; (B) the diabetic Langerhans islet with pleomorphic cells (green arrow), necrotic cells (red arrow) and infiltrated lymphocytes (yellow arrow); and (C) the recovery islet from stem cell-treated mouse with a few inflammatory cells (Magnification ×400, scale bar = 50 μm); (D, E, F): Immunofluorescent images of insulin-stained islets (Magnification ×200, scale bar = 50 μm).

Firstly, we successfully isolated and cultured umbilical cord derived UC-MSCs. These cells satisfied all criteria of MSCs that Dominici et al. have suggested (Dominici et al., 2006). Indeed, the cells exhibited the fibroblast-like shape when cultured as adherent cells in plastic flasks. They also expressed the MSC marker profile, which includes expression of CD44, CD73 and CD90, but absence of hematopoietic markers, e.g. CD34, CD45, CD14 and HLA-DR. With regard to the differentiation potential of these cells, we found that they could be successfully differentiated into mesoderm derived cells, including osteoblasts, chondrocytes and adipocytes.

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The UC-MSCs were used to treat T1D mice. The results showed that UC-MSC transplantation successfully rescued the pancreas which had been injured by STZ. Indeed, all treated mice exhibited an increase in body weight and decrease in blood glucose levels. These changes suggest that the metabolism of the UC-MSC treated mice significantly improved such that the mice could absorb the glucose and maintain water in their cells, leading to the increase in body weight. More importantly, the significant increase of insulin in peripheral blood confirmed that in the treated mice, beta cells could produce insulin again. The glucose tolerance test also supported this observation; treated mice could absorb glucose better than placebo mice. The results from H&E staining and anti-insulin antibody staining for islets emphasize again that treated mice successfully regain pancreatic function.

Successful regeneration of the pancreas is connected to UC-MSC transplantation. We hypothesized that grafted UC-MSCs might suppress pancreatic inflammation caused by STZ. Indeed, STZ contains a glucose molecule (in deoxy form) that is linked to a highly reactive methylnitrosourea moiety that is thought to exert the cytotoxic effects of STZ, while the glucose moiety directs the chemical to the pancreatic β cells (Johansson and Tjalve, 1978). STZ recognizes the GLUT2 receptor on β cell plasma membranes (Lenzen, 2008). There are some mechanisms that STZ use to cause toxicity on beta cells. For instance, one mechanism may be mediated by the DNA alkylating activity of its methylnitrosourea moiety (LeDoux et al., 1986; Wilson et al., 1988). The transfer of the methyl group from STZ to the DNA molecule causes damage resulting in the fragmentation of the DNA (Yamamoto et al., 1981). Moreover, STZ has potential to act as an intracellular nitric oxide (NO) donor (Turk et al., 1993) and is involved in the minor generation of ROS, including superoxide and hydroxyl radicals originating from hydrogen peroxide dismutation during hypoxanthine metabolism (Nukatsuka et al., 1990). All the aforementioned effects of STZ can cause beta cell death and significant injury to the pancreas. The injuries rapidly trigger the immune system to react to and attack the pancreas.

In the Type 1 diabetic mouse model (induced by STZ), transplantation of UC-MSCs can affect the T1D mice by at least 2 mechanisms. The first mechanism is suppression of inflammation as well as injury of islets related to STZ. The *in vitro* assays supported this mechanism. Immune cells (T cells) from murine peripheral blood could be suppressed by certain cytokines produced by UC-MSCs. This means that UC-MSCs can decrease or suppress the inflammation provoked by STZ damage to the pancreas.

In a previous study, we showed that UC-MSCs exhibited strong immune modulation capacity compared to bone marrow or adipose tissue derived mesenchymal stem cells (Pham et al., 2016). UC-MSCs could efficiently inhibit T cell proliferation via production of IFN-gamma, IL-1 beta, IL-2, and TNF-alpha; and PGE2 (Pham et al., 2016). The immune modulation of UC-MSCs on murine immune cells were also observed in other previously published studies (Chan et

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al., 2016; Fu et al., 2015). In endotoxin treated rats, Fu et al. (2015) demonstrated that the application of MSCs significantly reduced the levels of pro-inflammatory TNF-α and IL-1β, and increased the levels of anti-inflammatory IL-10 in these rats (Fu et al., 2015). Recently, Chan et al. (2016) showed that UC-MSCs effectively suppressed asthmatic symptoms, and that its immunomodulatory effect resulted primarily from suppressing the Th2 pathway in the animal model (Chan et al., 2016).

The second mechanism is related to paracrine effects of UC-MSCs. Some factors secreted by UC-MSCs can trigger the regeneration of the injured pancreas. In a previously published study by Zhou et al., the authors demonstrated *in vitro* that UC-MSCs could produce insulin-like growth factor 1 (IGF1) to support the islets (Zhou et al., 2015). UC-MSCs also provide a pool of varying kinds of growth factors, including matrix metalloproteinase-2, matrix metalloproteinase-9, hepatocyte growth factor, transforming growth factor β1, granulocyte-colony stimulating factor, fibroblast growth factor 2 and interleukin-6 (Santos et al., 2015). Based on these mechanisms, Zanier et al. have suggested that UC-MSCs can protect the murine brain after trauma (Zanier et al., 2011) and impact traumatic brain injury in humans (Wang et al., 2013).

Indeed, UC-MSCs can promote the regeneration of the injured pancreas. The role of pancreas regeneration also depends on the local pancreatic progenitor cells inside the pancreas. Via immunomodulatory factors secreted by UC-MSCs, pancreatic progenitor cells can be induced to proliferate and differentiate into beta cells which produce insulin, leading to an increase of insulin concentration in peripheral blood. In mice the islets of Langerhans (insulin positive cells) reappeared after 28 days of UC-MSC transplantation. The existence of pancreatic progenitor cells were confirmed by other studies (Hori et al., 2008; Oshima et al., 2007), suggesting that these cells could mature to become beta cells (Ma et al., 2012).

Conclusion

In this study, we show that UC-MSCs are easily isolated and cultured for transplantation applications. The cells maintained mesenchymal stem cell phenotypes after 5 passages in culture. Transplantation of these cells could significantly led to improvement in T1D mouse model of diabetes induced by STZ. UC-MSC treated mice recovered their body weight, blood insulin levels and blood glucose levels. More importantly, the function of pancreas was re-activated with insulin positive cells which existed in the mouse pancreases. This regeneration of injured pancreas was related to immune modulation and secreted trophic factors of UC-MSCs. Our findings suggest that UC-MSC transplantation is a suitable and promising treatment for type 1 diabetes mellitus.

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Abbreviations

BM: Bone marrow

GVHD: Graft-versus-host disease

H&E: Hematoxylin & Eosin

HLA: Human leukocyte antigens

HSCT: Hematopoietic stem cell transplantation

MSCs: Mesenchymal stem cells

STZ: Streptozotocin

T1D: Type 1 diabetes mellitus disease

UC-MSCs: Umbilical cord derived MSCs

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Author Contribution

NKP, BTBL isolated and confirmed UC-MSCs and wrote and revised the paper; the cell products have been prepared by TCNhat and BTVAnh; transplantation procedure and the monitor of body weight, blood glucose and glucose tolerance have been performed by NLTCong and BNTAnh; serum insulin measurement and pancreas structure evaluation carried out by DTTLoan and NLTCong.

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Case Report

Expanded autologous adipose derived stem cell transplantation for type 2 diabetes mellitus: a preliminary report of 3 cases and review of literature

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Abstract

Introduction: Type 2 diabetes mellitus (T2D) is the most common form of diabetes mellitus, accounting for 90% of diabetes mellitus in patients. At the present time, although T2D can be treated by various drugs and therapies using insulin replacement, reports have shown that complications including microvascular, macrovascular complications and therapy resistance can occur in patients on long term treatment. Stem cell therapy is regarded as a promising therapy for diabetes mellitus, including T2D. The aim of this study was to evaluate the safety and therapeutic effect of expanded autologous adipose derived stem cell (ADSC) transplantation for T2D treatment; the pilot study included 3 patients who were followed for 3 months. **Methods**: The ADSCs were isolated from stromal vascular fractions, harvested from the belly of the patient,and expanded for 21 days per previously published studies. Before transplantation, ADSCs were evaluated for endotoxin, mycoplasma contamination, and karyotype.All patients were transfused with ADSCs at 1-2x10^6 cells/kg of body weight.Patients were evaluated for criteria related to transplantation safety and therapeutic effects; these included fever, blood glucose level before transplantation of ADSCs, and blood glucose level after transplantation (at 1, 2 and 3 months). **Results**: The results showed that all samples of ADSCs exhibited the MSC phenotype with stable karyotype (2n=46), there was no contamination of mycoplasma, and endotoxin levels were low (<0.25 EU/mL). No adverse effects were detected after 3 months of transplantation. Decreases of blood glucose levels were recorded in all patients. **Conclusion**: The findings from this initial study show that expanded autologous ADSCs may be a promising treatment for T2D.

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Diabetes mellitus type 2, adipose derived stem cells,mesenchymal stem cells, stem cell therapy, autologous stem cell transplantation, stromal vascular fractions

Introduction

Diabetes mellitus (DM) affects approximately 300 million people worldwide (Scully, 2012). DM and its complications cause substantial morbidity and mortality in DM patients. Islet transplantation have been used to treat DM with some promising results (Hirshberg, 2007). Although these therapies can provide some benefit to patients there are limitations, namely the lack of pancreatic islets for transplantation (Hirshberg, 2007; McCall and Shapiro, 2012). In fact, two or three donors of pancreatic islets are required for a transplantation procedure for a single patient.

Recently, stem cell therapy has emerged as a highly promising new modality of treatment for advanced diabetes. For type 1 diabetes mellitus (T1D), both hematopoietic stem cell (HSC) transplantation and mesenchymal stem cell (MSC) transplantation have been evaluated (Cantu-Rodriguez et al., 2016; Cheng et al., 2016; El-Badawy and El-Badri, 2016; Snarski et al., 2016; Xiang et al., 2016; Xv et al., 2016). These studies have shown that HSC and MSC transplantations improve blood glucose levels from moderate to high. The meta-analysis study carried out by El-Badawy and El-Badri (2016) showed that the best therapeutic outcome could be achieved with CD34+ HSC transplantation, while the poorest outcome was observed with umbilical cord blood transplantation (El-Badawy and El-Badri, 2016).

Stem cell therapy has also been used to treat type 2 diabetes mellitus (T2D). Tong et al. (2013) infused umbilical cord blood by microcatheter into the dorsal pancreatic artery in 3 T2D patients with different diabetic histories (Tong et al., 2013). After the transplantation, Tong et al. showed that C-peptide levels increased in all of the patients; in fact reduced insulin dose corresponded with improved C-peptide levels (Tong et al., 2013). In 2014, 22 patients with T2D were treated with Wharton's jelly derived MSC (WJ-MSC) transplantation through intravenous injection and intra-pancreatic endovascular injection (Liu et al., 2014). Liu et al. showed that WJ-MSC transplantation significantly decreased the levels of glucose, improved C-peptide levels, and improved beta cell function, all without any adverse effects (Liu et al., 2014). In 2016, Hu et al. (2016) reported the long-term efficacy and safety of infusion of WJ-MSC on T2D in a study of61 patients with T2DM. After the 36-month follow-up period, infusion of WJ-MSC not only improved function of islet β-cells but reduced diabetic complications (Hu et al., 2016).

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Based on these results, we investigated the use of autologous expanded adipose derived stem cells (ADSCs) for transplantation to treat T2D patients. This study aimed to evaluate the preliminary outcome of autologous expanded ADSC transplantation, such as changes in blood glucose levels, at 1-3 months after transplantation.

Methods

Adipose tissue aspiration

Adipose tissues from patients were collected according to a previously published study (Nguyen et al., 2016). Briefly, patients were given local anesthesia with 2-3 mL of marcaine (5 g/L); the lower abdomen area was also anesthetized. Patients were then given a tumescent solution (500 mL normal saline, 0.5 mL of 1:1000 epinephrine). A Triport Harvester cannula (Becton Dickinson, Franklin Lakes, NJ) and 60-cc BD Luer-Lock™ syringe (Becton Dickinson) were used for harvesting adipose tissues (100–500 cc) from patients.

Isolation of stromal vascular fractions (SVFs)

The SVFs were isolated from collected adipose tissues. Approximately 100 mL of lipoaspirate was collected from each patient into two 50 ml sterile syringes. The syringes were stored in a sterile box at 2–8°C and immediately transferred to the laboratory. The SVFs were isolated using a Cell Extraction Kit (RegenmedLab, Ho Chi Minh, Vietnam) according to the manufacturer's instructions. Briefly, adipose tissues were digested using SuperDigest Solution (containing collagenase) included in the kit. The tissues were incubated in the solution at 37°C for 15 min with agitation at 5-min intervals. The suspension was centrifuged at 800×*g* for 10 min, and the SVFs were obtained as pellets. The pellet was washed twice with PBS to remove any residual enzyme, and re-suspended in PBS for determination of cell quantity and viability using an automatic cell counter (NucleoCounter; Chemometec, Lillerød, Denmark).

Activated platelet-rich plasma (PRP) preparation

Besides adipose tissue, activated PRP (aPRP) was prepared for each patient. PRP was isolated from peripheral blood using a kit (5PRP Kit, Regenmedlab, Ho Chi Minh, Vietnam) according to the manufacturer's guidelines. Briefly, 20 mL of peripheral blood was collected into vacuum tubes and centrifuged at 800×*g* for 10 min. The plasma fraction was collected and centrifuged at 1000×*g* for 5 min to obtain a platelet pellet. Most of the plasma was then removed, leaving 6 mL of plasma for re-suspension of the platelets. The inactivated PRP was then activated using activating tubes containing 100 µL of 20% $CaCl_2$.

Adipose derived stem cell culture

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SVFs were cultured for expansion according to published procedures (Van Pham et al., 2014). SVF samples were cultured in MSCCult medium (RegenMedLab, Ho Chi Minh, Vietnam) which contained DMEM/F12 supplemented with antibiotic and antimycotic solutions, 10 ng/mL epidermal growth factor (EGF), and 10 ng/mL basic fibroblast growth factor (bFGF) with 3% aPRP. The cells were plated at 5×10^4 cells/mL in T-75 flasks (Corning) and incubated at 37°C with 5% CO_2. After 3 days of incubation, 6 mL of fresh medium was added to each flask. After 7 days, the medium was replaced with 12 mL of fresh medium. The culture medium was subsequently replaced every 3 days until the cells reached 70–80% confluence, at which point they were sub-cultured. The samples were cultured for 21 days with 3 sub-cultures.

Preparation of product for injection

The product for injection was composed of a mixture of the collected ADSCs and activated PRP. Activated PRP was used to dilute ADSCs to achieve a suitable dose for injection with 10^7 SVF cells/mL.

ADSC transplantation and monitoring

Patients were transfused with 10^6 ADSCs/kg in 250 mL of saline through the arm vein. Patients were monitored for blood glucose levels for every 2 weeks. Moreover, for longer evaluation, patients were monitored for C-peptide and HAb1c up to 12 months after transplantation.

Case representation

Case report: Case 1

Patient 1 (NTKO, female, year 1981) was diagnosed as T2D for 2 years. Patient 1 had adipose tissue at belly collected to isolate and culture ADSCs. After 21 days, the cells were confirmed to be MSCs since they were negative for CD14 and CD45, and positive forCD44 and CD90. The cells were evaluated and shown to have no mycoplasma contamination and low endotoxin (<0.25 EU/mL), as well as stable karyotype (2n=46). These cells were then harvested and re-suspended into 250 mL of physiology saline. The patient was transfused with her ADSCs in saline through the arm vein at a dose of $1x10^6$ cells/kg for 30-45 min. Blood glucose was measured before transplantation and after transplantation (every 2 weeks until the report time). The results showed that the blood glucose level of Patient 1 gradually decreased after transplantation with her ADSCs, from 11.88 mM/L before transplantation to 9.85 mM/L, 10.89 mM/L, 9.49 mM/L and 7.84mM/L after 2, 4, 6, and 8 weeks of transplantation, respectively. There were no adverse effects recorded for Patient 1, especially hypoglycemia.

Case report: Case 2

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Patient 2 (LTL, female, year 1959) was diagnosed with T2D for 2 years. Similar to patient 1, adipose tissue was also collected from the belly of the patient to isolate and culture ADSCs. The collected ADSCs also exhibited typical MSC phenotype, e.g. positive for CD44 and CD90, and negative for CD14 and CD45. The cells were negative for mycoplasma and showed low endotoxin level (<0.25 EU/mL). The cells were transfused to patient 2 at $1x10^6$ cells/kg in 250 mL of saline for 30-45 min. No adverse effects was recorded in this patient during a 2-month follow up post transplantation. No attack of hypoglycemia was noted by patient and clinicians. Moreover, the blood glucose level gradually decreased from 8.04 mMl/L before transplantation to 7.05 mM/L and 7.07 mM/L after 2 weeks and 4 weeks of transplantation, respectively.

Case report: Case 3

Patient (DTL, female, year 1961) was confirmed as T2D for 2 years. The patient's ADSCs were isolated and expanded for treatment. ADSCs were confirmed as MSCs from positive markers (CD44 and CD90) and they were also negative for CD14 and CD45. They also maintained the normal karyotype with 2n=46. These cells also were not contaminated with mycoplasma and had low endotoxin (<0.25 EU/mL). The cells were transplanted into the patient at a dose of 10^6 cells/kg in 250 mL of saline for 30 min. Patient 3 was monitored for 12 months. To report time, there was no side effects recorded for this patient. The transplantation caused a decrease of blood glucose level from 9.35 mM/L (before transplantation) to 7.32 mM/L (4 weeks post transplantation).

Discussion

T2D is complex disease with various mechanisms which likely induce insulin resistance in patients. In this study, we show that autologous expanded ADSC transplantation can reduce insulin resistance in the T2D patients. The mechanism by which ADSCs could reduce insulin resistance has been investigated in this study. However, based on the literature of several previous studies noting the effects of ADSCs and T2D pathophysiology, we recognized that there were likely connections between the two. The first clue showing the connection of ADSCs and T2D mechanism is chronic inflammation. There has been increasing evidence showing that T2D is linked to chronic inflammation which causes insulin resistance. Antuna-Puente et al. (2008) and Sell et al. (2012) showed that chronic inflammation of adipose tissue significantly contributed to insulin resistance via adipokines (Antuna-Puente et al., 2008; Sell et al., 2012).

The accumulation of macrophages in some tissues (e.g. vasculature, adipose tissue, muscle and liver) can cause the chronic metabolic stress-induced inflammation (Bhargava and Lee, 2012). Moreover, macrophages play an important role in controlling the Th1/Th2 immune responses through the co-stimulating molecules CD80/CD86 and released cytokines (Bhargava and Lee,

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2012). These macrophages could produce inflammatory cytokines, such as IL-6 and TNFα, and induce insulin resistance in major metabolic tissues (Bhargava and Lee, 2012; Devaraj et al., 2010; Rajwani et al., 2012).

These observations were confirmed in other previously published studies (Kamei et al., 2006; Kanda et al., 2006; Patsouris et al., 2008). These studies showed that if depletion of $CD11c^+$ macrophages or inhibition of macrophage recruitment via MCP-1 knockout in obese mice could significantly reduce inflammation and increase insulin sensitivity (Kamei et al., 2006; Kanda et al., 2006; Patsouris et al., 2008). Increasing evidence in animals and humans about inflammation-induced insulin resistance in T2D were provided, and included abnormalities of lymphocytes (DeFuria et al., 2013; Winer et al., 2011), neutrophils (Talukdar et al., 2012), eosinophils (Wu et al., 2011), mast cells (Liu et al., 2009) and dendritic cells (Musilli et al., 2011). Therefore, the biggest challenge for treatment of T2D was modifying these immune cells to repair the insulin resistance.

ADSCs can participate in the correction of immune cells via their immune modulation capacity. In fact, while ADSCs can inhibit the proliferation of B cells, they can induce regulatory B cells (Franquesa et al., 2015) and induce functional de-novo regulatory T cells with methylated FOXP3 gene DNA (Engela et al., 2013). ADSCs can also successfully inhibit the allergic airway inflammation via immuno-modulation from a Th2 to a Th1-biased response in the mouse model (Cho and Roh, 2010). Moreover, ADSCs can inhibit some chronic inflammatory diseases of the CNS (Constantin et al., 2009).

ADSCs modulate the immune system via changes in anti-inflammatory cytokine expression and T-cell functions in hindlimb ischemia (Kuo et al., 2011). By this mechanism, ADSC transplantation could protect the nephrotoxic injury resulting in reduced kidney damage and functional improvement, inhibiting organ fibrosis and providing long-term immune regulation (Burgos-Silva et al., 2015; Kim et al., 2012). By this mechanism, ADSCs have not only been successful at treating diseases in animal models but also in humans. Recently, Stepien et al. (2016) used ADSCs to treat Multiple Sclerosis in human; they showed that intrathecal treatment of ADSCs was a suitable therapy for cases with aggressive disease progression (Stepien et al., 2016).

Hence, ADSC transplantation can modulate or correct the immune system, especially the chronic inflammation inside the T2D patients. Although, this report only introduces some results after 3 months follow up of 3 patients, the similar results after transplantation of ADSCs in all 3 patients shows that ADSC transplantation significantly reduces the insulin resistance.

In the previous clinical trials, using Wharton's jelly derived MSCs, Liu et al. (2014) showed that MSC transplantation significantly decreased the levels of glucose and glycated hemoglobin, while improving C-peptide levels and beta cell function (Liu et al., 2014). In another study, Jiang et al. (2011) used placenta

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derived MSCs to treat 10 T2D patients. The results showed that transplantation of MSC represents a simple, safe and effective therapeutic approach for T2D patients with islet cell dysfunction (Jiang et al., 2011).

Although this investigation was a short study, with only evaluation of blood glucose levels, these initial results show that autologous expanded ADSC transplantation may be a promising therapy for T2D. This study has been modified and is now monitoring out to 12 months post transplantation; blood glucose levels as well as C-peptide and hemoglobin A1c (HbA1c) concentrations are being evaluated.

Conclusion

Our study shows some initial results of autologous expanded adipose derived stem cell transplantation for T2D. We show that this is a safe and effective therapy for T2D. In all 3 patient cases in our study, transplantation of autologous ADSCs gradually decreased blood glucose levels up to 2 months, without any adverse effects or complications. Moreover, ADSCs could successfully be isolated and could proliferate during expansion. They showed normal MSC phenotype, exhibited stable karyotype, had no mycoplasma contamination and had low endotoxin. These preliminary findings show that autologous expanded ADSC transplantation is a promising therapy for T2D treatment. This study is in continuation to monitor up to 12 months post transplantation- to record changes in the levels of blood glucose, C-peptide and HbA1C.

Abbreviations

ADSC: Adipose derived stem cells
aPRP: activated platelet rich plasma
DM: Diabetes mellitus
HbA1C: Hemoglobin A1c
MSCs: Mesenchymal stem cells
SVF: Stromal vascular fraction
T2D: Type 2 diabetes mellitus

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Author Contribution

PTBL: prepared the patients, collected the adipose tissue, transplanted stem cells to the patients; PVP: collected the data, analysed the data, wrote the manuscript, suggested the treatment procedure; NBV, LTTD and NKP: cultured the stem cells, analysed the stem cells, prepared the stem cell products for transplantation.

Ethics approval

This study was approved by Ethical Comittee, Ministry of Health, Viet Nam.

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Original Research

Neuroprotective effects of herbal cocktail on cerebrovascular dysfunction in rats with induced hyperhomocysteinaemia

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Abstract

Introduction: Hyperhomocysteinaemia (HHcy) is an established risk factor for cardiovascular, cerebrovascular, peripheral vascular diseases and neurodegenerative disease. The effect of this HHcy on vascular diseases could potentially cause vascular pathology features. Experimental studies have demonstrated that Hcy can be neurotoxic to brain, hippocampus area. **Methods:** The present study was conducted to compare the possible neuroprotective effects of different herbal cocktail in HHcy-induced rats' brain cerebrovascular dysfunction model. Rats were divided into nine groups: Group I - Controls received the same volume of saline solution (0.5 mL/100 g of body weight). Group II served as HHcy and received homocysteine 0.03 µmol/g of b.w. daily for 30 days. Group III served as HHcy and received homocysteine 0.03 µmol/g of b.w. + Artemisia Judaica extract (AJ) (50 mg/kg per oral by oral feeding needle with tuberculin syringe) daily for 30 days. Group IV served as HHcy and received homocysteine 0.03 µmol/g of b.w.+ Panax ginseng extract (PG) (50 mg/kg per oral by oral feeding needle with tuberculin syringe) daily for 30 days. Group V served as HHcy and received homocysteine 0.03 µmol/g of b.w. + Polygonum multiflorum extract (PM) (400 mg/kg per oral by oral feeding needle with tuberculin syringe) daily for 30 days. Group VI served as HHcy and received homocysteine 0.03 µmol/g of b.w. + AJ + PG with the same dose of previous group daily for 30 days. Group VII served as HHcy and received homocysteine 0.03 µmol/g of b.w. + AJ + PM with the same dose of the previous group daily for 30 days. Group VIII served as HHcy and received homocysteine 0.03 µmol/g of b.w. + PG + PM with the same dose of the previous group daily for 30 days.

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Group IX served as HHcy and received homocysteine 0.03 µmol/g of b.w. + AJ + PG + PM with the same dose of previous group daily for 30 days. The hippocampus of brain samples was collected at the end of the experiment and measuring oxidative stress markers (CAT, SOD, MDA and NO), inflammatory mediators (IL-6 and BDNF), histopathological examination and comet assay. **Results:** Revealed data showed that the homocysteine induces SOD, CAT depletion, and an increase in AChE, MDA, NO, IL-6, and BDNF. A mixture of PG and PM or the individual treatments showed an ameliorative response for all parameters. In general, oxidative stress parameters, inflammatory mediator, neurotrophic factor, pathological examination and comet were degenerate against HHcy but did not differ significantly compared to AJ group. **Conclusion:** Better physiological and histological characteristics were in PG and PM and their combination groups compared with HHcy and ameliorated nearly the control group.

Keywords

Cerebrovascular Dysfunction, Hyperhomocysteinaemia, Herbal Cocktail, Rats

Introduction

Homocysteine (Hcy) is sulphur-containing amino acid derivative which causes damage to the endothelial cells of blood vessels and leads to atherosclerosis and vascular disorder (Kumar et al., 2008). Hyperhomocysteinaemia (HHcy) is recognised as one of the major risk factors for stroke and cerebrovascular disease (Boysen et al., 2003). This is supposed by the observation of McCully (1969) on autopsy of two infants born with homocysteinuria. They had died due to atherosclerosis of most of their blood vessels and myocardial disorder. These two infants had elevated levels of homocysteine, suggested cause of their death (Sainani et al., 2007). HHcy also acts as a cofactor in many cardiovascular, neurovascular and renal diseases; it causes remodeling of blood vessels and affects blood-brain barrier (BBB) permeability (Kumar et al., 2008).

Panax ginseng (pg) is widely used as a traditional herbal medicine. As major class of active ingredient that is responsible for the physiological activity of ginseng is the ginsenosides. It is not a single compound but a collection of different components (Algohary et al., 2016). Various studies recorded the beneficial effects of ginseng; constriction of toxins uptake, inhibition of excitotoxicity, reduction of oxidative stress, by controlling nitric oxide production and getting rid of free radicals (Van Kampen et al., 2014). Ginseng also had neuroprotective effect as recorded in various models like stroke (He et al., 2012), Alzheimer's disease (Kim et al., 2013), Parkinson's disease (Van Kampen et al., 2014), ALS (Khabazian et al., 2002), and Huntington's disease (Wu et al., 2009). Another study reported that ginseng was effective in the treatment of cardiovascular disease induced by HHcy (Kim et al., 2013).

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Polygonum multiflorum (pm) extract has been used as an anti-ageing herb in countries of East Asia. 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (THSG) is the main component of Polygonum multiflorum, its structure is similar to resveratrol. Many clinical studies have shown that Polygonum extract reduces progression of hypercholesterolaemia (Algohary et al., 2016), cardiac disease, neurosis and diseases commonly related to ageing (Lee et al., 2014). Polygonum extract has been reported to have various beneficial effects including antioxidant activity, anti-inflammatory and lipid metabolism regulation (Li et al., 2005). Another study revealed that Polygonum has neuroprotective effects against focal ischaemia, monoamine oxidase inhibitor, and improve learning and memory (Ling and Xu).

Genus of Artemisia (*Asteraceae, Anthemideae, Artmisiianae*) include about five hundreds of numerous species, spread all over world (geographical areas), and draw more attention due to high economic importance, especially medicinal properties (Ben-Nasr et al., 2013). The major bioactive composites of *Artemisia judaica* (AJ) essential oils (artemisyl-oil, apiperitone-oil, piperitone and trans-ethyl cinnamate), saponins, terpenes, tannins, and flavonoids (apigenin, cirsimaritin, flavonoid glycosides) which exhibit antioxidant, anti-inflammation and antihyperlipidaemic activities (El-Massry et al., 2002).

Artemisia judaica (AJ) is a permanent plant which spreads widely in the Sinai deserts of Egypt. Reasonably these plants were utilised for decades as, anti-venom, anti-anthelminthic, antidepressant, antiseptic, diuretic, antispasmodic, hypoglycaemic, anti-cancer, antihypolipidaemic, etc. (Ben-Nasr et al., 2013).

The aim of this study is to compare the possible neuroprotective effects of different herbal cocktail and select the best mixture in HHcy-induced rats' brain cerebrovascular dysfunction.

Methods

Experiments were performed in weanling Wistar rats, aged 21 days and weighing 40-50 g. They were housed in cages (6/cage) under controlled conditions. Animals were supplied from animal house of National Organization for Drug Control and Research (NODCAR). The animals were fed *ad libitum* and allowed to adjust to the new environment for two weeks before starting the experiment. The animals were housed at 22 ± 2^0C light/dark cycles. Animal use and care for experimental procedure were approved by the Institutional Animal Care and Use Committee (IACUC) of NODCAR.

Extract preparation

Approximately 150 g of *Artemisia Judaica* leaf were extracted twice with 70% ethanol using a 2 h. reflux extraction, and the extract was concentrated under

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reduced pressure. The concentrate was filtered, lyophilised, and subsequently stored at 4^0C. The yield of the dried extract from starting crude materials was 16.22% (w/w).

Approximately, 100 g of *Panax* ginseng root was extracted twice with boiling water, filtered, evaporated in a rotary vacuum evaporator, and freeze-dried. The yield of the dried extract from starting crude materials was 21.07% (w/w). Approximately, 1500 g of *Polygonum multiflorum* root was extracted twice with 70% ethanol using a 2 h. reflux extraction, and the extract was concentrated under reduced pressure. The concentrate was filtered, lyophilised, and subsequently stored at 4^0C. The yield of the dried extract from starting crude materials was 13.73% (w/w).

Experimental design

A total of fifty-four male rats (*Sprague-Dawley*) of 21 days old were utilised in this study and adapted in cages for one week. Rats were randomly divided into nine groups, each comprising six rats. The study was conducted for 30 days after 30 days age.

Homocysteine (Sigma-Aldrich®) (0.03 µmol/kg of b.w.) was administered subcutaneously, twice a day, from the 30^{th} to the 60^{th} day of the life of rats (Scherer et al., 2011). The rats were decapitated 12 h. after the last Homocysteine injection. The brain hippocampus weighed and kept frozen at -80°C until biochemical analysis.

Group I: Controls received the same volume of saline solution (0.5 mL/100 kg of b.w.).

Group II: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/kg of b.w. daily for 30 days.

Group III: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/kg of b.w. + *Artemisia judaica* extract (50 mg/kg b.w. by oral feeding needle with tuberculin syringe) daily for 30 days.

Group IV: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/kg of b.w. + *Panax* ginseng extract (50 mg/kg per oral by oral feeding needle with tuberculin syringe) daily for 30 days.

Group V: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/g of b.w. + *Polygonum multiflorum* extract (400 mg/kg per oral by oral feeding needle with tuberculin syringe) daily for 30 days.

Group VI: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/g of b.w. + *Artemisia Judaica* + *Panax* ginseng with the same dose of previous group daily for 30 days.

Group VII: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/g of b.w. + *Artemisia Judaica* + *Polygonum multiflorum* with the same dose of previous group daily for 30 days.

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Group VIII: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/kg of bodyweight + *Panax* ginseng + *Polygonum multiflorum* with the same dose of previous group daily for 30 days.

Group IX: served as hyperhomocysteinaemia and received homocysteine 0.03 µmol/kg of b.w. + *Artemisia Judaica* + *Panax* ginseng + *Polygonum multiflorum* with the same dose of the previous group daily for 30 days.

Determination of MDA by HPLC

Preparation of the standard solution

MDA standard was prepared by dissolving 25 µL 1,1,3,3-tetraethoxypropane (TEP) in 100 mL of water to give a 1 mM stock solution. Working standard was prepared by hydrolysis of 1 mL TEP stock solution in 50 mL 1% sulphuric acid and incubation for 2 h at room temperature. The resulting MDA standard of 20 nmol/mL was further diluted with 1% sulphuric acid to yield the final concentration of 1.25 nmol/mL to get the standard for the estimation of total MDA (Karatepe, 2004).

MDA activity was determined after extraction using following protocol: The samples were analysed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column Supelcosil C18 (5 µm particle and 80 A° pore size) (250 x 4.6 ID). Mobile phase consists of 30 mmol KH2PO4 and methanol (65%-35%, H3PO4 by pH 4), and the mobile phase at a 1.5 mL/min. flow rate, wavelength 250 nm according to the method of Karatas et al. 2002 (Karatas et al., 2002). Data Presented in **Fig.3**. The resulting chromatogram identified MDA position and concentration from the sample as compared to that of the standard as previously reported in monoamine content calculation.

Determination of nitrites and nitrates by HPLC

Nitrites and nitrate was determined according to the method of Papadoyannis, Samanidou, and Nitsos 1999 by HPLC (Papadoyannis et al., 1999).

Preparation of the standard solution

Sodium nitrite and sodium nitrate used for the reference standard preparation with stock concentration 1 mg/mL. A standard mixture of nitrite and nitrate was used to determine the retention times and separation of the peaks. Nitrite and nitrate concentrations were equal in the mixture solution.

NO was determined after extraction using following protocol: The samples were analysed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column was anion exchange PRP-X100 Hamilton, 150 x 4.1 mm, 10 µm. The mobile phase was a mixture of 0.1 M NaCl - methanol, at a volume ratio 45:55. The flow rate of 2 mL/min., wavelength adjusted to 230 nm. Data Presented in **Fig. 4**. The resulting chromatogram identified each of nitrite and nitrate

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positions and concentration from the sample as compared to that of the standards.

Determination of Superoxide dismutase (SOD) activity

The activity of SOD was determined according to the method of Marklund and Marklund 1974 (Marklund and Marklund, 1974). SOD activity was determined after extraction using following protocol: In a spectrophotometer Micro Cuvette, 0.985 of tris-HCL buffer was added to 0.010 mL of Pyrogallol solution and 0.005 mL of double distilled water. Absorbance was measured at 420 nm immediately and after one minute, the resultant ΔA was considered as the experimental blank. The same procedure was carried out by the use of 0.005 mL of the prepared SOD containing solutions (standard) or sample instead of double distilled water.

Determination of Catalase (CAT) activity in brain tissue homogenate

Catalase (CAT) activity was measured by the method of Del Maestro and McDonald 1987. The method is based on the rate of H_2O_2 degradation by the action of CAT contained in the examined samples followed spectrophotometrically at 230 nm in 5 mM EDTA, 1 M Tris-HCl solution, pH 8.0.

Determination of Acetylcholinesterase (AChE) activity in brain tissue homogenate

The procedure used for the determination of acetylcholinesterase activity in the brain hippocampus samples of rabbit is a modification of Ellman et al. 1961 (Ellman et al., 1961) method as described by Gorun et al. 1978 (Gorun et al., 1978).

AChE activity was determined after extraction using following protocol: The brain hippocampus tissue samples were weighed and homogenised in a 20-mmol-phosphate buffer, pH 7.6 (5 % w/v). The following reagents were pipettes in a cuvette: 0.14-mL phosphate buffer 20 mmol (pH 7.6), 0.05 mL of 5-mmol acetylthiocholine iodide and 0.01 mL of tissue homogenate or serum. After 10 min. of incubation at 38°C, the reaction was stopped with 1.8 mL of DTNB – phosphate ethanol reagent. The colour was read immediately at 412 nm using Shimadzu spectrophotometer UV –1601. Omitting the enzyme from the incubation mixture made the control samples. After addition of the colour reagent, appropriate amount of tissue homogenates or serum was added to the control. The cholinesterase activity was determined as μmol SH from a standard curve.

Determination of IL-6 in the brain

IL-6 levels in the rat brain were estimated using a rat-specific immunoassay kit (Rat IL-6 ELISA) from Glory Science (Del Rio, Texas, USA) according to the manufacturer's protocol. The intensity of the coloured product was directly

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proportional to the concentration of rat IL-6, as evaluated using a micro plate reader (Biotech ELx800; Biotech Instruments) set at 450 nm. The sample concentration was determined against a standard curve and is expressed in nanograms of IL-6 per gram of brain tissue.

Determination of BDNF in the brain

BDNF levels were estimated using a rat-specific immunoassay kit (Rat BDNF ELISA) from Glory Science, according to the manufacturer's protocol. The intensity of the coloured product was directly proportional to the concentration of rat BDNF, as determined using a micro plate reader (Biotech ELx800) set at 450 nm. The sample concentration was determined against a standard curve and is expressed in nanograms.

DNA Comet Assay

Comet assay of DNA was estimated according to the classic alkaline single-cell electrophoresis protocol (Xu et al., 2013). Samples were stained with SYBR Green I (Sigma-Aldrich, St Louis, MO) and analysed by Comet Score 1.5 software. Percent of DNA in comet tails was considered as the marker of genotoxic effect.

Statistical analysis

The values were expressed as the mean ± SE for the 6 male rats in each group. Differences between groups were assessed by one way analysis of variance (ANOVA) using SAS (2004) software for Windows (version 13.0). Statistical analysis of the obtained data was performed using the general linear model (GLM). Significant differences among means were evaluated using Duncan's (1955) (Duncan, 1955). Multiple Range Test.

The following linear model was applied: $Yij = \mu + \alpha i + \xi ij$, Yij = Observation measured, M = Overall mean, αi = Effect of treatment. ξij = Experimental error assumed to be randomly distributed ($\sigma 2 = 0$).

Results

The effect of some herbal extracts on nitric oxide (ACHE), catalase (CAT), malondialdehyde (MDA) and superoxide dismutase (SOD) in hyperhomocysteinaemic rats

Data presented in **Fig. 1, 2, 3** and **4** records the effect of homocysteine oral administration on antioxidant parameter (CAT, MDA, NO and SOD) of hippocampus brain area at the end of experiment. Homocysteine induces a significant decrease ($p<0.05$) in the hippocampus CAT and SOD and significant increase ($p<0.05$) of NO and MDA compared to control group. On the other

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hand, PG, PM, AJ+PG, PG+PM and AJ+PG+PM showed significant differences ($p<0.05$) against homocysteine group and rounded than the control group. Also, AJ+PM showed ameliorative response and nearly rounded to control for CAT, NO, MDA and SOD compared with homocysteine group but AJ only didn't show any improvement than homocysteine group.

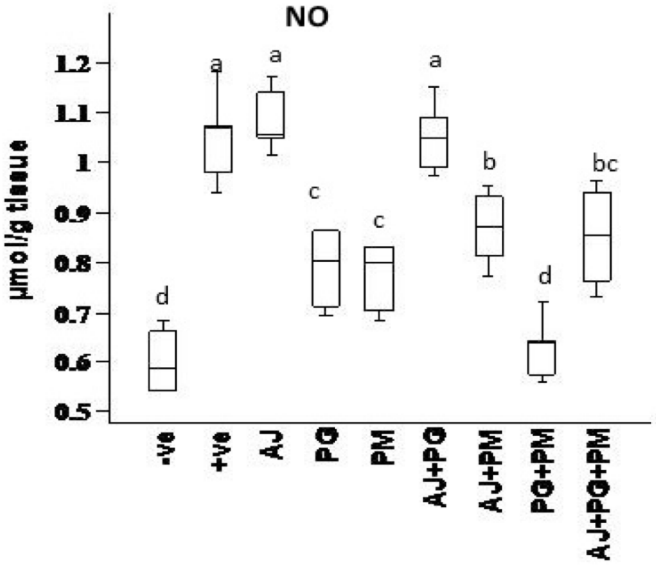

Figure 1. The effect of some herbal extracts on nitric oxide (NO) in hyperhomocysteinemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

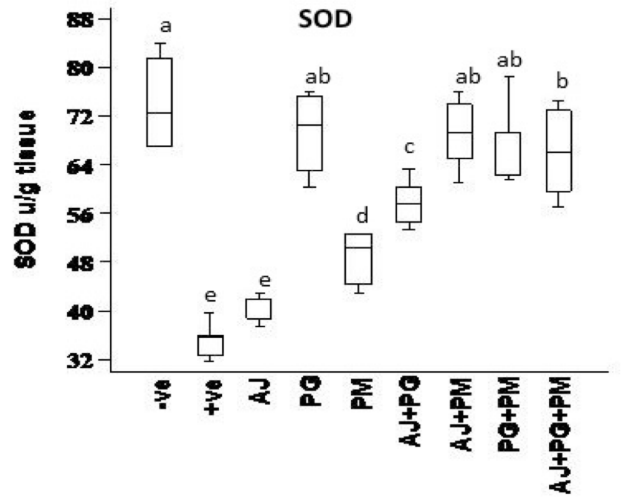

Figure 2. The effect of some herbal extracts on catalase (CAT) in hyperhomocysteinemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

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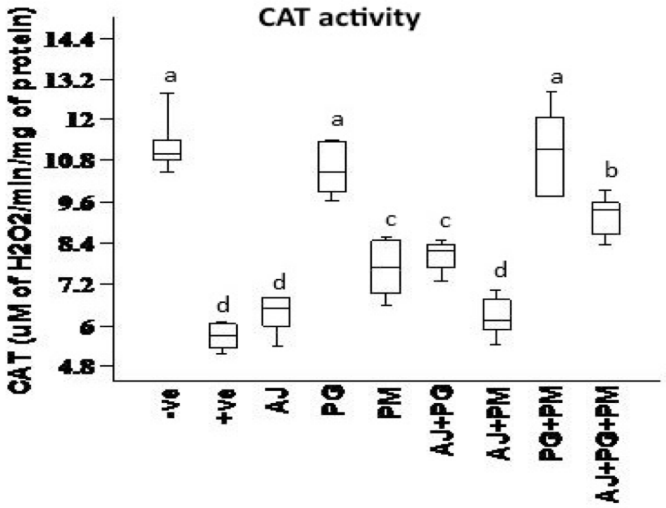

Figure 3. The effect of some herbal extracts on malodialdhyde (MDA) in hyperhomocysteinemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

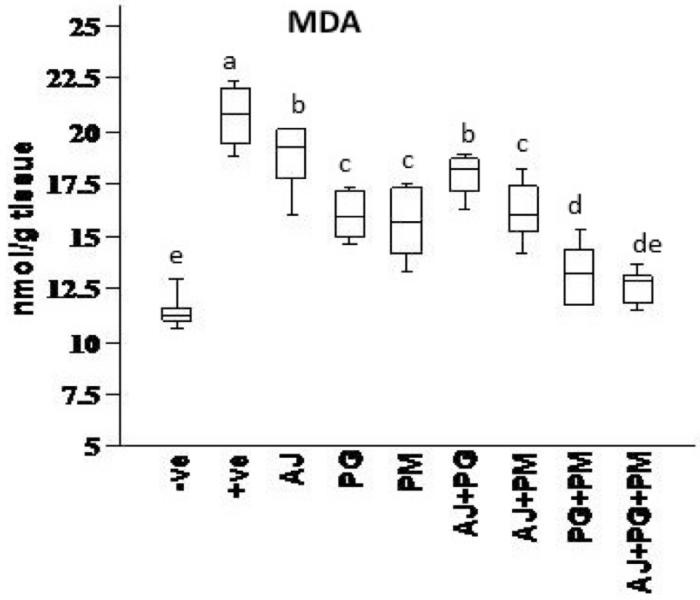

Figure 4. The effect of some herbal extracts on superoxide dismutase (SOD) in hyperhomocysteinemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

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The effect of some herbal extracts on (ACHE), (IL6) and (BDNF) in hyperhomocysteinaemic rats

As showed in **Fig. 5, 6,** and **7**, records the effect of homocysteine oral administration on IL-6, BDNF and SOD of hippocampus brain area at the end of experiment. Homocysteine caused elevation in inflammatory markers (IL-6), and AChE of hippocampus brain area. Homocysteine also induces a significant decrease ($p<0.05$) in the hippocampus neurotrophic factor (BDNF) as compared to control group. On the other hand, all treated group except AJ only showed significant ($p<0.05$) ameliorative effect of SOD, IL-6, and BDNF against homocysteine group and rounded than the control group, but AJ only didn't show any improvement than homocysteine group at the same parameters.

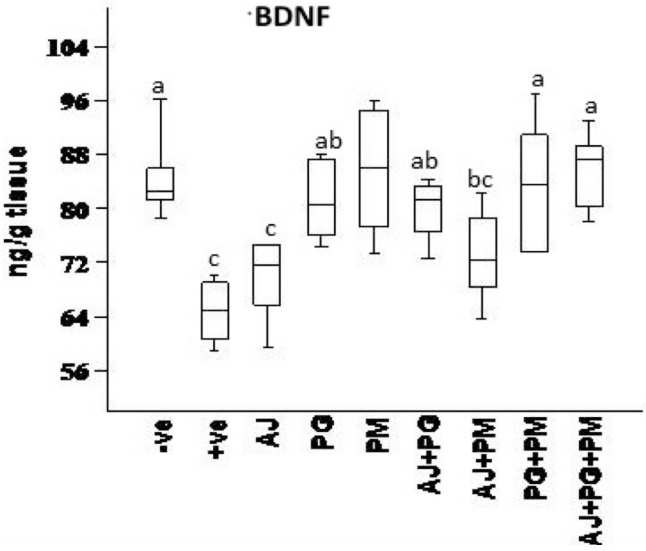

Figure 5. The effect of some herbal extracts on (AChE), in hyperhomocysteinaemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

Concerning the brain genotoxic potential of homocysteine using the comet essay, there was a significant increase in the tail length of DNA, tail intensity (DNA%) and tail moment in the homocysteine-treated rats compared to the control. On the other hand, the PG and PM treated groups significantly decreased DNA tail length, intensity and moment as compared to the homocysteine-treated rats, while the AJ treated groups showed no pronounced effect (**Table 1** and **Fig. 8**).

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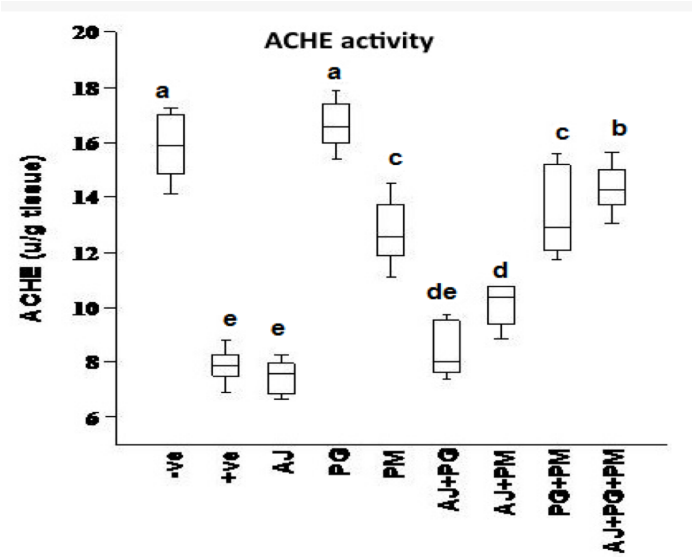

Figure 6. The effect of some herbal extracts on (IL6) in hyperhomocysteinaemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

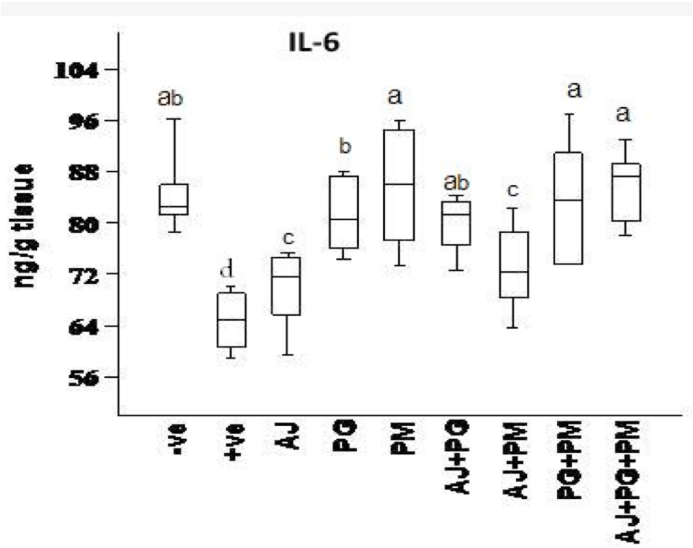

Figure 7. The effect of some herbal extracts on (BDNF) in hyperhomocysteinaemic rats. Data are expressed as Mean ± S.E. for 6 rats/ group/ a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).

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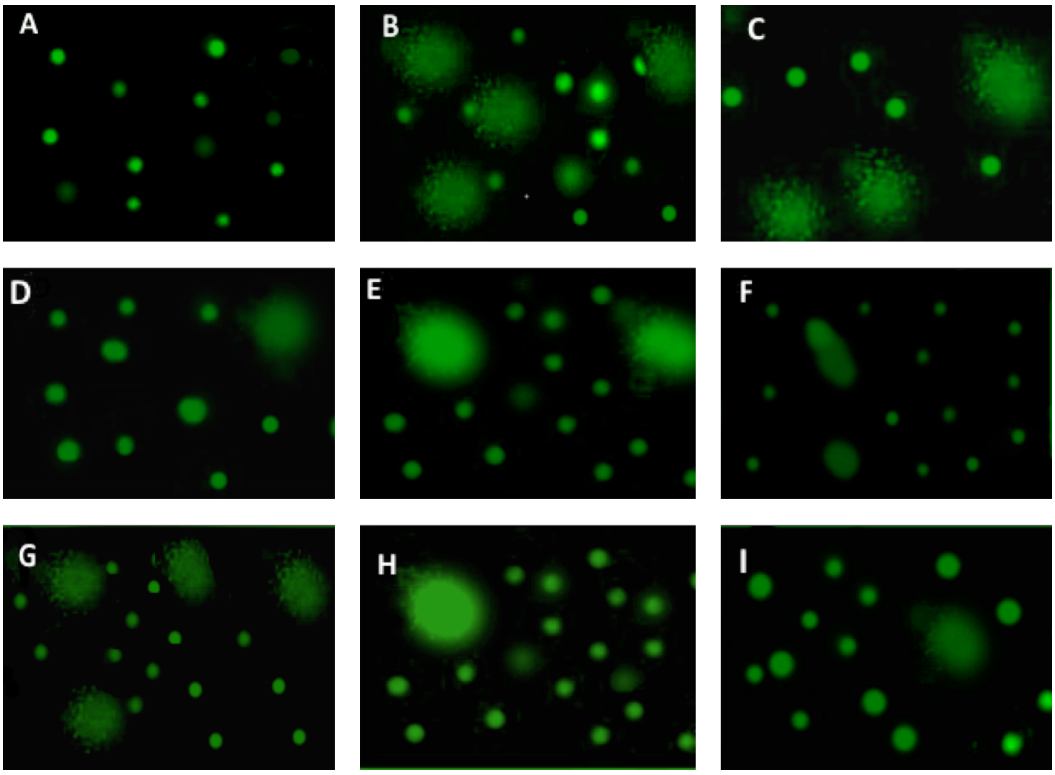

Figure 8. The effect of some herbal extracts on comet score in hyperhomocysteinaemic rats. Photographs showing the effect of herbal cocktail treatment against homocysteine-induced DNA damage in the hippocampus of rat as measured by the comet assay represented by Tail length (μm) and % of DNA damage in the brain cells from control (a), +ve control rats (B), AJ treated rats (C), PG treated rats (D), PM treated rats (E), AJ+PG treated rats (F), AJ+PM treated rats (G), PG+PM treated rats (H), and AJ+PG+PM treated rats (I).

Discussion

Stroke, one of the most risks of severe disability and death, data from numerous studies concluded that stroke and ischaemic disease accompanied by elevated homocysteine level (Boysen et al., 2003).

Hyperhomocysteinaemia caused endothelial dysfunction by the low density lipoproteins direct oxidation, its cytoplasmic oxidation of compounds like homocysteine, mixed disulphides and homocysteine thiolactone, which lead to the release of free radicals as reactive oxidative species ROS (hydrogen peroxide, superoxide anion and hydroxide radical) and acceleration of fibrin and collagen accumulation in endothelia (Plazar and Jurdana, 2012). In present study, homocysteine caused elevation in oxidative stress (MDA and NO), and inflammatory markers (IL-6) of hippocampus brain area. Homocysteine also induces a significant decrease ($p<0.05$) in the hippocampus CAT, SOD,

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neurotrophic factor (BDNF) and significant increase ($p<0.05$) of AChE compared to control group. Another study suggested that HHcy caused elevation of oxidative stress markers, impaired endothelium vasorelaxation and suppression endothelial nitric oxide synthase (Zhou et al., 2005).

Table 1. The effect of herbal cocktail treatment against homocysteine-induced DNA damage in the control and experimental groups

	T. DNA%	T. Moment (units)	T. Length μm
-ve	1.36 ± 0.14^{c}	1.09 ± 0.18^{e}	1.40± 0.06^{c}
+ve	4.11 ± 0.09^{a}	15.04 ± 0.50^{a}	3.72 ± 0.06^{a}
AJ	3.94 ± 0.15^{a}	14.73 ± 0.37^{a}	3.57 ± 0.03^{a}
PG	2.70 ± 0.26^{b}	6.60 ± 0.23^{bc}	2.73 ± 0.08^{b}
PM	2.72 ± 0.07^{b}	8.03 ± 0.49^{b}	2.63 ± 0.07^{b}
AJ+PG	2.71 ± 0.15^{b}	5.93 ± 0.05^{c}	2.59 ± 0.07^{b}
AJ+PM	2.68 ± 0.08^{b}	7.90 ± 0.06^{b}	2.46 ± 0.07^{b}
PG+PM	1.49 ± 0.04^{c}	1.99 ± 0.15^{d}	1.79 ± 0.06^{c}
AJ+PG+PM	1.43 ± 0.10^{c}	1.94 ± 0.16^{d}	1.77 ± 0.03^{c}

- *Data are expressed as Mean ± S.E. for 6 rats/group*
- *a, b, c, d, e means having different superscript letters in the same column differ significantly ($p<0.05$).*

On the other hand, the recent study extends to evaluate the neuroprotective effects of some herbal extracts. The protective effects of PG, PM, AJ and its mixtures against hyperhomocysteinaemia are investigated, PG, PM, AJ+PG, PG +PM and AJ+PG+PM showed notable differences against homocysteine group and rounded than the control group. Also, AJ+PM showed ameliorative response and nearly rounded to control for SOD and AChE compared with homocysteine group, but AJ only didn't show any improvement as compared to homocysteine group, all treated group also showed significant differences of MDA against homocysteine group and rounded than the control group. Also, all treated group except AJ only showed vital effects of NO, IL-6, and BDNF against homocysteine group and rounded than the control group, but AJ only didn't show any improvement than homocysteine group at the same parameters.

Oral administration of *PG* could modulate the toxic effect of homocysteine. Several studies suggested that the antioxidant and vasorelaxation effect of ginseng is the key role in its neuroprotective effect. Bioactive components of PG

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(ginsenosides) have high antioxidant activity and blocking the oxidation of lipids (Zhou et al., 2005). These phenolic compounds such as flavonoids, phenolic acids, diterpenes, saponins, and tannins could reduce cell damage induced by oxidative stress (Algohary et al., 2016). In recent study, PM extract treatment alone also showed a protective effect against hyperhomocysteinaemia. This is in agreement to several studies that suggested PM had protective effect against ageing and other diseases commonly related to ageing, as stroke, ischaemia, hyperlipidaemia, coronary heart disease, neurosis (Lee et al., 2014). This may be due to Stilbene glycoside, the bioactive compound of PM. It is similar to resveratrol, exhibits many pharmacological properties, including antioxidant, anti-inflammatory, and lipid regulator (Li et al., 2005). Stilbene, Resveratrol, and other polyphenolic compounds may also increase nitric oxide bioavailability, thereby supressing the progress of endothelial dysfunction; reducing blood viscosity, improving insulin sensitivity, counteracting platelet hyperactivity, suppressing platelet adhesion to fibrinogen-coated surfaces (Malinowska and Olas, 2011). Stilbene glycoside also caused significant improvement in learning and memory by reduction of MDA, and monoamine oxidase in the cerebral cortex. Another study revealed that stilbene glycoside inhibits microglial inflammation by releasing of proinflammatory markers as TNF-α, IL-1β, IL-6, and NO (Ling and Xu, 2016).

The co-administration of PG and PM in the recent study enhanced there vital effects, as compared to its individual treatment.

Conclusion

In conclusion, the present study suggests that herbal cocktail (mixture of PG 50 mg/kg b. wt. and PM 400 mg/kg b. wt. may reduce the toxic effects of Hcys by decreasing oxidative stress, releasing pro-inflammatory mediators, increasing NO bioavailability and inhibiting the progress of endothelial dysfunction. Therefore, may be potentially useful in the prevention of hyperhomocysteinemia and other related cerebrovascular diseases.

Abbreviations

AChE: Acetylcholinesterase
AJ: Artemisia Judaica extract
ANOVA: Analysis of variance
BBB: blood-brain barrier
BDNF: Brain-derived neurotrophic factor
CAT: Catalase
GLM: general linear model

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HHcy: Hyperhomocysteinaemia
IACUC: Institutional Animal Care and Use Committee
IL-6: Interleukin - 6
MDA: Malondialdehyde
NO: nitric oxide
NODCAR: National Organization for Drug Control and Research
PG: Panax ginseng extract
PM: Polygonum multiflorum extract
SOD: Superoxide dismutase
TEP: 1,1,3,3-tetraethoxypropane
THSG: 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside

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Author contribution

Dr. A.M. Algohary and Dr. R. Al-Baradie contributed in paper with phyto analysis and extraction (40%). Dr. O.A. Ahmed-Farid and Dr. A.M. Abd-Elrazek contributed in paper with biological modeling and evaluation in animal of all extraction (60%).

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