INTRODUCTION

Allogeneic haematopoietic stem cell transplantation (HSCT) from an HLA matched sibling donor is the optimal treatment option for patients with severe aplastic anaemia (AA). Over the last few decades, outcome of AA has improved considerably with up to 80% long-term survival, although graft rejection, GVHD and infectious complications remain serious obstacles. Graft rejection and poor survival after HSCT for AA has been linked to pre-transplant blood transfusions, an interval greater than one month between diagnosis and transplant, androgen treatment before transplant, and delays between diagnosis and transplant lead to acquisition of multiple poor risk factors. AA is the most frequent indication for allogeneic HSCT in AA patients undergoing allogeneic haematopoietic stem cell transplantation.

METHODOLOGY

This is an analysis of 125 consecutive AA patients who underwent HLA identical sibling stem cell transplants at the Armed Forces Bone Marrow Transplant Centre, Rawalpindi, from July 2001 to June 2010. Patients having a minimum of 100 days follow-up post-transplant were considered, while patients surviving at least 21 days post-transplant were included in the analysis for rejection and GVHD. All patients had bone marrow aspiration and trephine biopsy to confirm diagnosis of AA and were categorized as per Camitta's criteria. Fanconi anaemia was excluded by stress cytogenetic analysis using mitomycin C. All patients had six antigen matched siblings or family donors. Informed written consent was obtained from all patients.

Three conditioning regimens were used: (a) cyclophosphamide (Cy) 50 mg/kg for 4 days (day-6 to -3) and anti-lymphotye globulin (ALG) 15 mg/kg or ATG (Fresenius) at 3.75 mg/kg for 3 days (day-5 to -3), (b) campath-1H 20 mg/day for 5 days on day-9 to-5 with Cy 50 mg/kg for 4 days (day-6 to -3) and (c) fludarabine 30 mg/m², Cy 300 mg/m² and ATG 3.75 mg/kg for four days (day-6 to-3). Bone marrow harvested from iliac bones under general or spinal anaesthesia was the stem cell source alone or with G-CSF mobilized peripheral blood stem cells (G-PBSC) collected by aphaeresis. Fourteen patients received PBSC alone. Stem cell dose was calculated as total nucleated cell (TNC) dose for bone marrow and as mononuclear cell (MNC) dose for PBSC. The target MNC/TNC dose was > 3.0x10⁹/kg of recipient body weight.

GVHD prophylaxis was with intravenous ciclosporin-A (CSA) starting on day-2 at 5 mg/kg given in two divided doses daily and reduced to 3 mg/kg from day+5 onwards. Target CSA blood level was 200-300 ng/ml. Prednisolone 1 mg/kg body weight was given from day+1 and tapered off over 4-6 weeks. Most patients also received short methotrexate (MTX) at following doses: 10 mg/m² on day +1 and 8 mg/m² on day+3 and +6. After encountering high incidence of mucositis, folinic acid 15 mg/m², 6 hourly for four doses 24 hours after each MTX dose was given routinely to all patients. Prior to discharge from hospital intravenous CSA was replaced with oral formulation that was continued for 12 months and then tapered off to stop at 15 months post-transplant if there was no GVHD. In patients with poor tolerance to CSA, mycophenolate mofetil was introduced with CSA dose reduction.

All patients were kept in single rooms with positive pressure high efficiency particulate air (HEPA) filters. Ciprofloxacin, fluconazole and acyclovir were started on day-2 as antibacterial, antifungal, and antiviral prophylaxis respectively. *Pneumocystis* prophylaxis was with nebublised pentamidine on day-1; oral Co-trimoxazole was started post-transplant once the neutrophil count was > 1.5 x 10⁹/l. Fluconazole was stopped at 3 months, while acyclovir and co-trimoxazole continued till 12 months post-transplant. All febrile episodes were treated using intra-venous piperacillin-tazobactam and amikacin with or without teicoplanin as first line antibiotics. Amphotericin B or other antifungals were given if fever persisted for more than 3-5 days on first line antibiotics. Haemoglobin was maintained above 8 g/dl and platelet count above 10 x 10⁹/l with irradiated, leuko-depleted red cell concentrates and platelet transfusions.

Neutrophil and platelet engraftment were defined as the first of three consecutive days with an absolute neutrophil count > 0.5 x10⁹/l and a platelet count > 20 x 10⁹/l respectively. For ABO mismatched transplants, donor's blood group was used to document and follow engraftment. For others short tandem repeat (STR) analysis by PCR was carried out.

Statistical calculations were done using Statistical Package for Social Sciences (SPSS) version 17.0 (Chicago, Illinois, USA). Estimation of overall survival (OS) included all patients who were alive on the date of last evaluation while disease-free survival (DFS) was estimated as survival in the absence of death or rejection. Median follow-up days were calculated for the survivors. The log-rank test was used for univariate analysis to assess the significance of differences in survival for various factors like gender, age, time from diagnosis to transplant, stem cell source, stem cell dose, occurrence of acute or chronic GVHD, number of red cell and platelet transfusions before transplant. Variables with p-value of < 0.1 in univariate analysis were included in the multivariate analysis. For all tests a p-value of 0.05 or less was taken as statistically significant.

**RESULTS**

Demographics of the patients and donors with disease characteristics are listed in Table I. All patients received grafts from sibling donors except for 2 patients who received bone marrow from HLA matched cousins. Record for pre-transplant red cell and platelet transfusions was available for only 36 and 33 patients respectively. In these patients, the median number of red cell transfusions was 4 units (range 2-100 units) and platelet transfusions were 50 events (range 1-250). Five patients stopped taking immunosuppressants on their own for various reasons (2 due to financial reasons, 2 due to severe depression and a teenage girl due to behaviour issues). Two of these patients rejected their graft and died due to sepsis while 3 patients died of severe acute GVHD. These 5 patients were excluded from analysis of rejection, GVHD, DFS and OS. Various post-transplant complications are given in Table II.

A total of 12 patients had graft rejection (10.5%). One patient had primary engraftment failure and underwent second transplant from the same donor after conditioning with Flu/Cy/ALG. Eleven patients experienced graft rejection after having engrafted initially: four of these patients died of infective complications; one patient did not opt for a second transplant and is alive with stable disease 284 days post-transplant, while 6 patients received a second transplant from the same donor. Four of these patients achieved long-term engraftment, one patient rejected her second graft 5 weeks after transplant and is alive with partial autologous recovery and one patient died of septicaemia during second transplant. Factors associated with graft rejection were female gender (7 out of 29 female patients vs. 5 out of 85 male patients, p = 0.006) and pre-transplant disease duration of > 9 months (5 out of 26 patients with disease duration > 9 months vs. 7 out of 88 with disease duration < 9 months, p = 0.1).
Twenty out of 113 evaluable patients (17.6%) had acute GVHD. All patients but one had acute skin GVHD grade I-II and responded to glucocorticoids and intensification of immunosuppression. One patient had grade IV GVHD involving the skin, gut and liver, and died on day+84 post-transplant. Patients who received CSA+MTX+prednisolone for GVHD prophylaxis had less acute GVHD than patients who did not receive MTX (9 out of 75 patients vs. 11 out of 38 patients, p= 0.026). Male gender was associated with greater acute GVHD (19 out of 85 vs. 1 out of 28 patients, p=0.024). There was no statistically significant effect of patient or donor age, donor to recipient gender mismatch or conditioning regimen on the incidence of acute GVHD. Fifteen patients (13.5%) developed chronic GVHD. Thirteen patients (11.3%) had limited chronic GVHD (6: oral mucosa, 5: hepatic, 2: skin); 2 patients (1.8%) had extensive chronic GVHD involving the skin, eyes and buccal mucosa. Chronic GVHD was seen more frequently in patients > 20 years of age (10 out of 35 vs. 5 out of 76 patients, p = 0.002), with donors > 20 years of age (11 out of 38 vs. 4 out of 73 patients, p = 0.001) and with Flu/Cy/ATG conditioning (8 out of 28 vs. 7 out of 77 patients, p = 0.02). There was no effect of patient gender, disease duration, gender mismatch or GVHD prophylactic regimen on occurrence of chronic GVHD. Majority of patients experienced chills and fever during ALG/ATG infusion but there was no anaphylaxis. One patient had mild VOD which was controlled with fluid restriction and diuretics. Mucositis was a significant problem in patients who received MTX as part of GVHD prophylaxis. Two patients who underwent ABO mismatched transplants developed pure red cell aplasia 8 and 9 weeks post-transplant respectively. Both were treated with steroids and rituximab. The first patient responded with normalization of haemoglobin and haematocrit, while the second patient showed a partial response and is still receiving low doses of CSA and prednisolone 25 months post-transplant. Fifty patients had neutropenic fever for 3 days or more. Focus of infection could be identified in 14 patients, while 10 patients had positive cultures. A total of 19 patients (15.8%) died of various complications given in Table II. The median follow-up was 1185 days (range 111-3356) with overall survival (OS) and disease-free survival (DFS) of 84.2% and 78.3% respectively (Figures 1 and 2).
Following factors (given in Table III) were identified to be associated with a superior OS in univariate analysis: male gender ($p < 0.001$), Flu/Cy/ATG conditioning regimen ($p = 0.198$) and use of bone marrow as the stem cell source ($p = 0.317$). In univariate analysis, three major factors associated with better DFS were male gender ($p < 0.001$), Flu/Cy/ATG conditioning ($p = 0.515$) and pre-transplant disease duration 9 months or less ($p = 0.04$). On multivariate analysis, female gender was found to be predictor of poorer OS and DFS ($p = 0.001$).

**DISCUSSION**

HSCT is the standard curative treatment for SAA in young patients with reported long-term survival of more than 80% and graft failure rates of about 10%. A higher incidence of the disease among younger patients and a uniformly poor response to immunosuppressive therapy (unpublished data from this centre) make HSCT the only practical treatment option. Majority of the Pakistani population lives in communities of large families, where inter-marriages are common and population growth is high. Hence the likelihood of finding a fully matched sibling donor can be as high as 60-70%. This advantage is offset by endemic poverty, a shortage of health care and transfusion services and a lack of awareness leading to delayed referral for transplant. Therefore, a majority of patients receive multiple transfusions, some of which may be directed donations from close family members. Leukocyte filters are rarely used. Many patients are given steroids, androgens or CSA and they have had several episodes of sepsis. Many of these factors are common in other developing countries and lead to a poor outcome with graft rejection rates as high as 31%.

At a median follow-up of 1185 days (range 111-3356) OS and DFS of our patients was 84% and 78% respectively. Male gender and Flu/Cy/ATG conditioning regimen were associated with a superior OS and DFS. A majority of these patients were given Cy+ALG/ATG conditioning. It has been a standard conditioning regimen for allogeneic HSCT in AA associated with improved survival and low graft rejection rates by several investigators; although a prospective randomized study showed no improvement in outcome when this regimen was compared with Cy
Female gender was found to be predictor of poorer OS and DFS (p = 0.001) on multivariate analysis. This finding cannot be explained as there was no significant difference between male and female patients when other variables such as patient age, disease severity, disease duration and pre-transplant transfusions were compared. Another local study on 100 cases undergoing SCT for various disorders showed female gender as predictor of poor survival. Male to female ratio in these patients was 3:1. Since there is no significant difference in incidence of AA between males and females, this may be reflective of the gender bias inherent in the local culture where males are brought to medical attention more readily than females.

G-PBSCs with or without bone marrow was used as the stem cell source in majority of these patients in order to enhance the stem cell dose and reduce time to neutrophil recovery. These patients had a lower OS but similar DFS as patients who received bone marrow alone. Patients who received G-PBSC also had more acute and chronic GVHD than those who received bone marrow (p=0.09 and 0.08 respectively). This is in agreement with a retrospective analysis of data from EBMT and CIBMTR which showed higher rates of chronic GVHD and overall mortality after transplantation with G-PBSC than with bone marrow in younger patients although there was no statistically significant effect on GVHD or OS for older patients. Other investigators have documented higher rates of chronic GVHD in patients of all age groups. On the other hand, in a study, using G-PBSC as the main graft source, George et al. have reported no increase in the risk of GVHD or an inferior survival but the median follow-up was only 22 months, and with longer follow-up the incidence of chronic GVHD may increase. Likewise, a preliminary study of transplant data from Pakistan on 20 HLA identical sibling PBSC transplants for AA showed 81% event-free survival.

Primary and late graft failure is a major factor contributing to increased morbidity and mortality after transplantation for AA and rejection rates as high as 30% have been reported. An EBMT analysis of SAA patients undergoing matched-related donor transplants showed a graft rejection rate of 12%. Multiple transfusions, episodes of sepsis before transplant and multiple treatments pre-transplant have all been seen to increase graft rejection. Out of 114 evaluable patients, 12 had graft rejection (10.5%) including one primary engraftment failure. Pre-transplant disease duration of more than 9 months was associated with increased graft rejection and lower DFS. Greater interval between diagnosis and transplant has been associated with more graft failure. Although majority of these patients had received multiple blood transfusions, incomplete pre-transplant transfusion data precluded any meaningful analysis of this factor. Two additional points are worth mentioning: firstly, more female than male patients had graft rejection, secondly, more graft rejection was seen with patients receiving Flu/Cy/ATG conditioning regimen. Five out of 7 females with graft rejection had a male donor. The effect of donor and recipient gender mismatching on transplant outcome was analyzed by EBMT; it was seen that female patients with male donors had a higher risk (22%) of graft rejection. Three different conditioning regimens were used and although there was no statistically significant effect of any particular regimen on graft rejection it was seen that out of the 79 evaluable patients who received standard Cy/ATG (or ALG) regimen, 6 patients (7.5%) experienced graft rejection. Twenty-nine patients received Flu/Cy/ATG which has been used for high risk AA patients and for matched unrelated donor transplants; 4 of these patients (13.8%) experienced graft rejection. This effect probably contributed to the similar DFS achieved by both regimens. With camPATH 1-H/Cy conditioning, 2 out of 6 patients (33.3%) had graft rejection after initial engraftment (day+30 and day+45 respectively). A high graft failure rate (24%) has been reported by Gupta et al. by using Cy and anti-CD 52 monoclonal antibodies with increase in both primary and secondary graft failure.

GVHD is a major obstacle to successful transplant outcome. The incidence of both acute and chronic GVHD was low in our patients (17.6% and 13.5% respectively). Only one patient developed grade IV acute GVHD and 2 had extensive chronic GVHD. Patients who had received MTX in addition to CSA and prednisolone had a lower incidence of acute GVHD.
(p=0.026) but there was no effect on chronic GVHD. This is in agreement with previous reports which showed a reduction in acute GVHD with CSA and MTX but no effect on chronic GVHD.\(^{25}\) None of these patients with acute or chronic GVHD experienced graft rejection.

**CONCLUSION**

The outcome of these patients with AA compared favourably with other centres. Flu/Cy/ATG conditioning regimen, bone marrow as stem cell source and CSA, prednisolone with short methotrexate as GVHD prophylaxis were associated with better survival in AA.

**REFERENCES**

