

Chronic Myeloid Leukemia Blood Inflicted Injury in Cord Derived Wharton's Jelly Mesenchymal Stem Cells

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ABSTRACT

Objective: To determine the effects of blood from CML patients on human umbilical cord derived Wharton's jelly mesenchymal stem cells (WJMSCs) for evaluation of their therapeutic potential.

Study Design: An experimental study.

Place and Duration of Study: Centre for Research in Molecular Medicine, University of Lahore, from September 2013 to December 2014.

Methodology: Possible behavior of WJMSCs in CML patients was assessed by culturing these cells in their plasma. WJMSCs at passage 3 were cultured in plasma isolated from 9 CML patients as well as 9 normal subjects. Effects on cell viability, proliferation, LDH release, paracrine factors (p38 and p53) and oxidative stress were evaluated.

Results: WJMSCs cultured in plasma of CML patients showed decreased viability, slow proliferation, high LDH release, high expression of p38 and p53 and a high oxidative stress compared to normal subjects.

Conclusion: Stressed environment of CML patients' blood/plasma induced injury to WJMSCs as well as reduced their viability. Effectiveness of these cells for therapeutics of CML is, therefore, likely to be reduced.

Key Words: Chronic myeloid leukemia. Wharton's jelly mesenchymal stem cells. Oxidative stress. Cell death. Proliferation.

INTRODUCTION

Chronic myeloid leukemia (CML) represents 20% of all adult leukemia cases. CML represents hyper-clonal proliferation of immature blood cells as well as a high degree of oxidative stress.^{1,2} Therapeutic options for CML include homoharringtonine, polyethylene glycol interferon, oral cytarabine, decitabine, growth factor modulation, imatinib mesylate and bone marrow transplantation. Imatinib mesylate, an oral Bcr-Abl Kinase inhibitor, has shown better results but presents a high frequency of cytogenetic and haematological remissions and patients invariably undergo disease progression in advanced phase following a brief period of relief.³

Human bone marrow transplants have been used for years as promising therapy but were not reported to be successful in many cases due to less availability and occurrence of graft-versus-host disease (GVHD). Cord blood units (CBU) replaced the hematopoietic stem cells but still immune rejection was observed in some cases. Promising results have been found in treatment of resistant GVHD by infusion of mesenchymal stem cells (MSCs).^{4,5} Some groups recommend the expansion of CBU stem cells leading to co-culture with MSCs.^{4,6}

Umbilical cord (UC) as a source of stem cells is of increasing interest for cell therapies as it presents little

ethical consideration and comprises immune privileged cells, which may be suitable for allogeneic based therapies.⁷ It has been shown that UC matrix, represented by the Wharton's jelly (WJ), surrounding umbilical vessels, contains a great number of mesenchymal cells.⁸ Wharton's jelly umbilical cord derived mesenchymal stem cells (WJMSCs) have many advantages like being an abundant resource of stem cells, ease of accessibility, lack of ethical issues and painless procedures for harvesting.⁹ WJMSCs have been proven to be valuable in cancer,¹⁰ both *in vivo* and *in vitro*.¹¹ WJMSCs have been reported to inhibit growth of human myeloid leukemia cells by inducing cell shrinkage and blebbing leading to death *in vitro*.¹²

The rationale in the present study that stressed environment in blood of CML patients may affect the therapeutic potential of WJMSCs, *in vitro*.

The objective of this study was to determine the effects of blood from CML patients on human umbilical cord derived Wharton's jelly mesenchymal stem cells (WJMSCs) for evaluation of their therapeutic potential.

METHODOLOGY

Blood from 9 registered CML patients (2 females and 7 males) and 9 normal persons (2 females and 7 males) were collected with the patients' consent. All patients and donors were HCV, HBV and HIV negative. This study was approved by the institutional Ethics Committee and the Biosafety Board at The University of Lahore, Lahore, Pakistan. Mothers selected for the study were negative for human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV). Five

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umbilical cords were obtained with the consent of the parents from full term caesarian sections.

The cord pieces were incubated in 3 mg/mL collagenase solution in a T75 flask (Invitrogen Inc., USA). After 3 hours, Dulbecco's modified eagle medium low glucose (DMEM LG) (Sigma Aldrich, USA) with 10% fetal bovine serum (FBS) (Sigma Aldrich, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma Aldrich, USA) was added to the T75-flask (Corning, USA) containing collagenase solution and cord pieces (Invitrogen Inc., USA). The medium was renewed after every 3 days. Cells were used at passage 3 for all further experiments.

Characterization was performed by immunocytochemistry. Cells were cultured for 24 hours in 6-welled plates, leading to removal of medium and washing with normal saline. The cells were then fixed with ice cold methanol for 20 minutes (Invitrogen Inc., USA), washed and non-specific binding was blocked with 1% BSA (Invitrogen Inc., USA) for 30 minutes. Permeabilization was performed for OCT4 by adding 0.1% Tween 20 (Invitrogen Inc., USA). The cells were then incubated with primary antibodies, i.e. CD49d, CD73, CD90, CD105, OCT4, SSEA4, CD34 and CD45 (Santa Cruz Biotechnology, USA) in a dilution of 1:100 for 1 hour at 37°C. The cells were then incubated in flouochrome conjugated secondary antibodies, i.e. FITC conjugated donkey anti-goat for CD105, SSEA4, OCT4 and SSEA4 (Santa Cruz Biotechnology, USA) while Texas red conjugated donkey anti-mouse for CD49d, CD73, CD90, CD34 and CD45 (Jackson Immunoresearch Laboratories, Inc. USA), in a dilution of 1:100 for 2 hours at 37°C. The cells were incubated with DAPI for nuclear staining. Images were captured on Flويد cell imaging station (Life Technologies, USA) for analysis of immunoflouresence.

Plasma was isolated from blood of normal and CML persons by adding 2U/mL heparin and subsequently, centrifuging at 10,000g. WJMSCs were plated at a density of 100,000 cells per well of a 6-well plate (Corning, USA) and cultured for 24 hours and subsequently, treated with DMEM LG along with 10% human plasma from normal persons (Group 1) or from CML patients (Group 2). After 3 days of culturing, medium was saved at -80°C for LDH assay, ELISA and oxidative stress, while cells were used for MTT assay and cells viability assay.

Cells after treatment were stained with crystal violet (Invitrogen Inc., USA) for 20 minutes. After washing with normal saline, color taken by cells was solubilized with dimethylsulfoxide (DMSO) (Invitrogen Inc., USA) and absorbance of solution was measured at 540 nm.

To compare the proliferative potential of both groups, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. Monolayer of cells was first washed with phosphate buffer saline (PBS) (Invitrogen Inc., USA). Five hundred µL complete

medium along with 60 µL MTT solution (Invitrogen Inc., USA) was added to cells and incubated for 2 hours at 37°C. Purple color crystals formed within cells were solubilized with DMSO and absorbance was taken at 570 nm.

LDH assay was performed using 5 µL medium from each group at the end of treatment using LDH assay kit (AMP Diagnostics, Austria), according to manufacturer's instructions. Briefly, 5 µL cell culture medium of both groups was mixed with 95 µL working reagent, incubated for 5 minutes and then absorbance was recorded at wavelength of 340 nm.

Solid phase sandwich ELISA was performed for p53 and p38. A microtiter plate (Corning, USA) was coated with primary antibodies, i.e. mouse anti p53 and rabbit anti-p38 (Invitrogen Inc., USA) and incubated for 48 hours at 4°C. After washing thrice with tris buffered saline (TBS), 100 µL cell culture medium from both groups was loaded into coated wells and incubated for 18 hours. Wells were then washed 3 times, blocked for one hour with 10% bovine serum albumin (BSA) leading to overnight incubation with secondary antibody, i.e. horse raddish peroxidase (HRP) conjugated goat anti-rabbit for p38 (Santa Cruz Biotechnology, USA) and HRP conjugated goat anti-mouse for p53. After washing, 100 µL chromogenic solution, 3,3',5,5'-tetramethylbenzidine (TMB, Invitrogen Inc., USA) was added leading to 0.1 mM HCl addition to stop the reaction. Using a microtiter plate reader, absorbance was measured at 450 nm, keeping 650 nm as reference value.

For this, 0.5 mL culture medium was added in a tube along with 2.0 mL disodium hydrogen phosphate buffer (0.3 M) and 0.25 mL 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB (0.001 M, Invitrogen Inc., USA). Mixture was incubated for 15 minutes and absorbance was measured at 412 nm.

Briefly, 0.1 mL culture medium was mixed with 1.2 mL sodium pyrophosphate buffer (52 mM, pH 8.3), 0.1 mL phenazinemetosulphate (PMS, 186 µM, Santa Cruz Biotechnology, USA), 0.3 mL nitrobluetetrazolium (NBT, 300 µM, Invitrogen Inc., USA) and reaction was started by addition of 0.2 mL nicotinamide adenine dinucleotide (NADH, 750 µM, SantaCruz Biotechnology, USA). After incubation at 30°C for 90 seconds, reaction was stopped by the addition of 0.1 mL glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 mL n-butanol. Mixture was incubated for 10 minutes and centrifuged at 2000 g for 5 minutes. Upper n-butanol layer was taken and its absorbance was recorded at 560 nm.

Culture medium 0.1 mL was taken and mixed with 1.0 mL phosphate buffer (10 mM, pH 7.0) and 0.4 mL H₂O₂ (0.2 M) (Sigma Aldrich, USA). Reaction was stopped by adding 2.0 mL dichromate acetic acid reagent. Samples were incubated for 10 minutes in a boiling water bath, cooled and absorbance was measured at 530 nm.

Level of malondialdehyde (MDA), a free radical species, was evaluated by measuring thiobarbituric acid reactive substances. For this, 0.2 mL cell culture medium was added to 0.2 mL sodium dodecylsulfate (SDS, 8.1%), 1.5 mL thiobarbituric acid (TBA, 0.8%), 1.5 mL acetic acid (20%, pH 3.5) and volume was made up to 4.0 mL with distilled water and incubated at 90°C for 60 minutes. After cooling, 1.0 mL distilled water, 5.0 mL n-butanol-pyridine mixture (15:1) was added and the mixture was shaken vigorously and centrifuged at 4000g for 10 minutes. Upper n-butanol layer was taken and its absorbance was measured at 532 nm.

Three independent experiments in triplicate were conducted for obtaining quantitative data. Statistical analysis was performed using GraphPad Prism version 5.00 for windows (GraphPad Software, San Diego California, USA). All results were expressed as mean ± standard deviation (SD). Student's t-test was used for comparison. Statistical significance was considered as $p < 0.05$.

RESULTS

The cultured WJMSCs expressed mesenchymal stem cells markers CD49d (56.56 ±6.26), CD73 (98.00 ±2.34), CD90 (99.33 ±1.11), CD105 (97.89 ±2.47) and embryonic markers OCT4 (45.89 ±7.20), SSEA4 (96.67±4.47), whereas no expression of hematopoietic stem cells markers CD34 and CD45 was observed by immunocytochemical analysis (Figure 1).

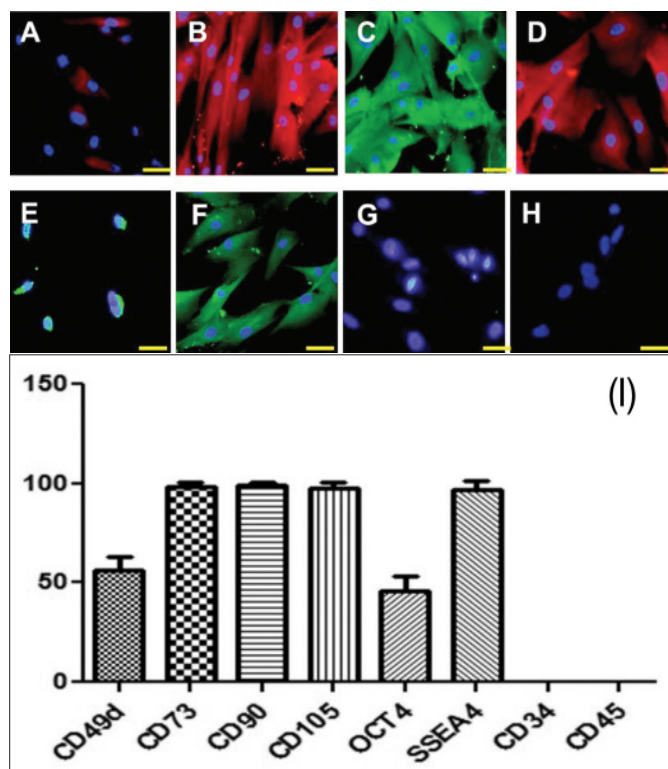


Figure 1: Characterization of cord derived stem. (A-H) immunocytochemistry for CD49d, CD73, CD90, CD105, OCT4, SSEA4, CD34, CD45, respectively (200X, scale bar 50 µm). (I) Graphical representation for percentages of positive cells for different markers.

Cells of Group 1 have more intense colour with crystal violet and higher absorbance value, hence more viability (Figures 2A and 2B). Cells cultured in CML plasma showed a significantly lower proliferation (0.12 ±0.04) compared to the control group (0.28 ±0.03) as estimated by MTT assay ($p < 0.001$, Figure 2B). Cytotoxicity was analyzed by LDH release which was significantly higher in the medium of Group 2 compared to Group 1 (Figure 2B).

Increased expression of p53 and p38 was observed in Group 2 (Table I). Antioxidant enzymes, i.e. level of GSH, SOD and catalase activities were significantly lower and MDA level was higher in Group 2 compared to Group 1 (Table II).

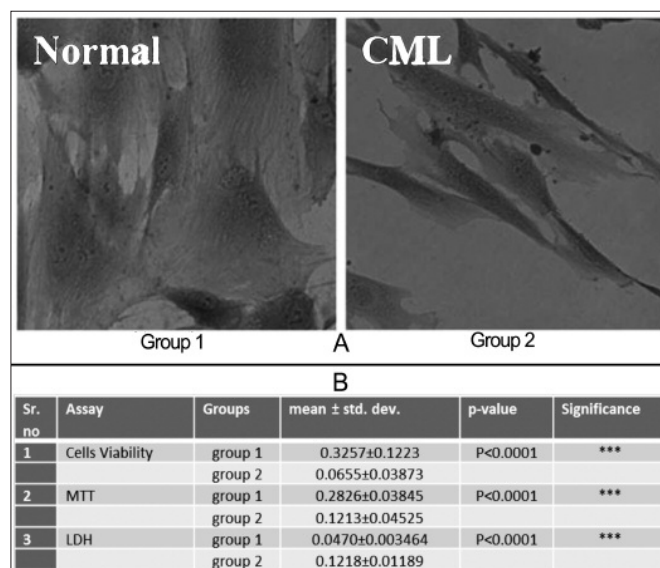


Figure 2: Effect of treatment of CML patients' plasma on growth of WJMSCs (Group 2) as compared to WJMSCs treated with normal persons' plasma (Group 1). (A) Microscopical representation of both groups (200X, scale bar 50 µm). (B) Table showing data of cells viability, MTT cells proliferation and LDH assay.

Table I: Gene expression analysis in WJMSCs treated with plasma from CML patients (Group 2) as compared to WJMSCs treated with plasma from normal persons (Group 1).

Assay	Groups	Mean ±std. dev.	p-value	Significance
P53 ELISA	Group1	0.0042 ±0.0005545	0.0003	***
	Group2	0.009533 ±0.003466		
P38 ELISA	Group1	0.03956 ±0.006447	p < 0.0001	***
	Group2	0.08706 ±0.01610		

Table II: Estimation of oxidative stress in both study groups.

Assay	Groups	Mean ±std. dev.	p-value	Significance
GSH	Group1	0.02822 ±0.004147	p < 0.0001	***
	Group2	0.01144 ±0.002833		
SOD	Group1	0.005444 ±0.002555	0.0221	*
	Group2	0.002778 ±0.001856		
Catalase	Group1	0.01611 ±0.004226	p < 0.0001	***
	Group2	0.04889 ±0.003257		
MDA level	Group1	0.002778 ±0.001394	0.0001	***
	Group2	0.006333 ±0.001581		

DISCUSSION

Stem cells therapy is a recent trend in treatment of various cancers including CML. Umbilical cord (UC) is considered a better option due to immune evasion properties.⁷ WJMSCs, the cells derived from umbilical cord, have been proven to induce apoptosis and lower the proliferation of CML cells *in vitro*.¹² The present study reveals the effects of CML plasma on the growth of WJMSCs to estimate potential behavior of WJMSCs in CML patients.

Cells were isolated from human umbilical cord tissue as according to the method of Pereira *et al.*¹³ The cells were attached to the surface of the culture flask and demonstrated morphology of mesenchymal stem cells. MSCs express CD10, CD13, CD29, CD44, CD49d, CD73, CD90, and CD105.^{14,15} This study revealed positive expression of mesenchymal markers CD49d, CD73, CD90, CD105 and embryonic markers OCT4 and SSEA4 with no expression of hematopoietic markers CD34 and CD45.

For transplantation a high dose of nucleated cells from umbilical cord blood (UCB) is required which is considered the only most significant factor that influences time to hematological reconstitution and chances of survival.¹⁶ This high dose may be related to the stress induced reduction in viability of cells. Viability of WJMSCs in CML plasma was assessed by staining cells with crystal violet, which has been used previously for assessment of cell's viability.¹⁷ An increased viability was observed in WJMSCs cultured in normal plasma as compared to WJMSCs cultured in plasma of CML patients. Proliferation of cells in both treatment Groups was assessed by MTT assay. MTT enters in actively metabolizing viable cells and it is converted into a purple color formazan product.¹⁸ MTT assay has been used to assess the viability and proliferation of human periodontal ligament stem cells.¹⁹ This study revealed a decreased rate of proliferation in WJMSCs in CML patients' plasma compared to control group. LDH release was significantly higher in the medium of WJMSCs treated with CML patients' plasma than in control group. It is reported that an elevated release of LDH by cells is an indicator of cytotoxicity.²⁰

p38 is considered as a protein involved in apoptosis,²¹ and p53 activates genes for apoptosis and cell cycle arrest.²² These results show increased expression of p38 and p53 proteins in Group 2, indicating apoptosis of WJMSCs in CML. Oxidative stress is characterized by excessive production of cellular ROS and/or defects in antioxidants. Multiple cellular processes involve the role of GSH like growth, apoptosis, chemo resistance, cancer progression as well as defence against oxidative stress. SOD and catalase are also considered important antioxidant enzymes and their reduced activity has been reported in cancer patients. High level of MDA indicates

lipid peroxidation, leading to oxidative stress. MDA level has been reported to be elevated in cancer conditions.²³

In CML, chronic oxidative stress has been evidenced, which suggests its role in disease progression.²⁴ The present results indicate increase in MDA (a free radical species) while significant decrease in GSH, SOD and catalase (antioxidant enzymes) in cells treated with CML plasma. These results show a marked oxidative stress in Group 2.

Limitations of the present study include the low numbers of subjects. The study also lacks the evaluation of possible mechanisms or ligands in blood of CML patients responsible for inflicting injury to cord derived stem cells. Efficiency of WJMSCs in animal model for CML must be tested and certain conditions should be provided to WJMSCs for improving their survival for therapy of CML.

CONCLUSION

This study reveals that stressed environment of CML blood induces injury to WJMSCs and reduces their viability. Efficacy of these cells for therapeutics of CML is reduced in cancer conditions, which compels further studies to improve their survival in such circumstances.

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