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Genomic insult oriented mitochondrial instability and proliferative hindrance in the bone marrow of aplastic mice including stem/progenitor population

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ABSTRACT

Aplastic anemia is the bone marrow failure condition characterized by the development of hypocellularity in both marrow and peripheral blood compartments. Anti-tumor chemotherapeutic agents often exert secondary effect on hematopoietic system leading to aplastic anemia by marrow failure. The precise mechanisms behind the marrow ablative effects of the drugs remain yet to be established. The present study holds a mechanistic approach to unveil the mystery. Aplastic anemia was generated in mice with the administration of busulfan and cyclophosphamide followed by the characterization of the disease with peripheral blood hemogram, histopathological and cytochemical examinations of bone marrow. To gain deep knowledge about the molecular mechanisms of the hematopoietic disruption, cytotoxicity assay, DNA damage measurement, apoptosis study, replicative senescence analysis, redox balance study, mitochondrial membrane potential change assessment, flowcytometric expressional analysis of p21, p53, ATM, Chk-2, Necdin, Gfi-1, c-myc, KU-80 and Sod-2 were done with marrow hematopoietic stem/ progenitor cells (HSPCs). Severe blood pancytopenia and marrow hypocellularity was found in aplastic mice. Proliferative hindrance and apoptosis of marrow cells were identified as the cause behind the hematopoietic catastrophe. The genotoxic effects of the drugs triggered chromatin damage and induced replicative senescence in aplastic HSPCs by upregulating p21 in a p53 independent manner. Moreover, accumulation of genomic insults also caused apoptotic elimination of marrow cells due to disruption of mitochondrial membrane potential by generating redox imbalance. The study established the underlying mechanisms behind hematopoietic disruption during drug induced marrow aplasia. Outcome of the study may be helpful in successful designing of therapeutic strategies for the disease concerned.

1. Introduction

Use of chemotherapy is one of the most common anti-neoplastic therapeutic modalities however the effect is not always tumor specific. Normal tissues, particularly bone marrow is susceptible to the cytotoxic insult [1–3]. Persistent myelosuppression or bone marrow failure is a common side effect of chemotherapeutic drugs which involves the damage in hematopoietic stem cell pool, progenitor cells as well as mature populations [4–11]. The severe catastrophe arising due to the destruction of blood forming cells in bone marrow often leads to the development of aplastic anemia characterized by hypoplastic marrow and peripheral blood pancytopenia [12–14]. The annual frequency of aplastic anemia or bone marrow aplasia is nearly 2 per million populations and its occurrence is about 2–3 times more in Asia than the west [15–17].

The pathophysiological pathways leading to medullary aplasia are often triggered by the accumulation of DNA damaging effect of genotoxic agents [18–20]. Despite the wide use of drugs viz; busulfan, cyclophosphamide etc. as potent chemotherapeutics and bone marrow transplantation preconditioning agents, the mechanisms by which they affect bone marrow causing aplastic anemia have not been well established [21–28]. Several recent works with animal models as well as patient case studies have identified two culpable factors behind hematopoietic catastrophe during aplastic anemia [12,29–33]. Firstly, there are evidences suggesting the overshoot of apoptosis of marrow or programmed cell death in aplastic condition. Secondly, initiation of premature senescence was reported in hematopoietic cells as evidenced by the occurrence of the symptom of replicative stress such as cell cycle arrest.

The present study was designed to determine the mechanistic

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Table 1
Comparative hemogram profile of control and aplasia groups. # marked values are significantly different from the control values.

Parameters	Control group (X ± SD)	Aplasia group (X ± SD)	P-value
WBC ($\times 10^3$/ mm^3)	8.43 ± 0.55	3.59 ± 0.6	P = 0.0005
RBC ($\times 10^6$/ mm^3)	7.98 ± 0.47	5.51 ± 0.57	P = 0.0045
Platelets ($\times 10^3$/ mm^3)	440.08 ± 8.23	214.36 ± 32.12	P = 0.0003
Hemoglobin (gm/dl)	13.85 ± 1.2	8.83 ± 1.84	P = 0.0168
Reticulocyte (%)	1.47 ± 0.47	0.32 ± 0.06	P = 0.0145
Neutrophils (%)	25.67 ± 2.52	8 ± 2	P = 0.0007

correlation between the etiologies of drug induced bone marrow aplasia with the induction of apoptosis and premature senescence in marrow cells using common murine model of aplastic anemia [5,30–32,34,35] generated by the administration of busulfan (BU) and cyclophosphamide (CP). The outcome of the study may pave the way into new horizon of cell based therapies for the dreadful disease concerned.

2. Materials and methods

2.1. Mice

Inbred Swiss albino mice (*Mus musculus*) of both sexes (8 weeks old; weighing 22–25 g m) were housed six per cage at animal room of Calcutta School of Tropical Medicine. Animals were given proper care viz; maintenance of sterility, temperature, humidity, 12 h dark-light cycle etc. They received adequate food and water ad libitum. The Institutional Animal Ethical Committee (IAEC) has approved all the experimental procedure used in the study.

2.2. Drug administration

Use of chemotherapeutic drugs like busulfan (BU), cyclophosphamide (CP) for developing bone marrow failure models is in practice for decades [5,31,36–42].

Two groups of animals (each containing 30 mice) were maintained for experimental purpose:

Group-I: Mice received two doses of each BU (20 mg/kg body weight) and CP (80 mg/kg body weight) intraperitoneally (i.p) at an interval of 28 days. Aplastic anemic (AA) condition appeared 12 weeks post second dose as confirmed by peripheral blood status.

Group-II: Control group of mice received similar doses of saline.

Animals were sacrificed post 12 weeks of second dose after confirming the aplastic features from peripheral blood analysis in experimental group.

2.3. Histological study

The long bones were harvested from both the groups of mice immediately after sacrifice. Bones were fixed with 10% buffered formalin for 24 h, decalcified with 10% formic acid, dehydrated by passing through ascending alcohol grades, embedded in paraffin and finally cut into 5 µm thick sections. Routine H & E staining was performed on each tissue. Bone marrow (BM) cellularity was assessed by visual examination under light microscope (Olympus, Japan). A tissue was considered hypocellular when < 30% of inter-trabecular space occupied by hematopoietic tissue [43].

2.4. Isolation of bone marrow cells and single cell preparation

Marrow cells were flushed out from bones into RPMI-1640 (Sigma, USA) using 26 gauge needle and syringe. Cells from 3 to 10 mice were pooled and made into single cells by repeat pipetting. Cells were washed thrice in ice-cold media to remove debris and finally passed through 100 µm cell strainer to obtain pure single cell suspension.

2.5. Oil red O staining

In order to assess the fat cell population, BM smears of control and aplastic mice were subjected to Oil Red O staining [44]. Briefly, air dried BM smears were fixed with 10% formaldehyde for 10 min, washed with Oil Red O solution (Oil Red O : water; 3:2) for 15 min at room temperature. Excess stain was washed off and smears were counter stained with Harris Hematoxiline for microscopic study. Red colored cells were identified as adipocytes.

2.6. Cellular replicative potential assessment

Proliferative potential of BM cells from control and aplasia groups were compared by BrdU incorporation assay according to manufacturer`s protocol (Invitrogen, USA) as described in our previous publication [42]. In brief, BrdU solution was given as i.p. injection (1 ml/100 gm body weight) in mice from both the groups. Animals were sacrificed 18 h post BrdU injection after which BM cells were isolated and made into single cells as mentioned earlier. Then 2 µl of FITC conjugated anti-BrdU antibody (Biolegend, USA) was added per 2×10^6 BM cells and incubated at 37° C for 30 min in dark. Finally, the cells were subjected to flowcytometric analysis.

2.7. In vitro evaluation of cytotoxic insult of BM cells by aplastic stress

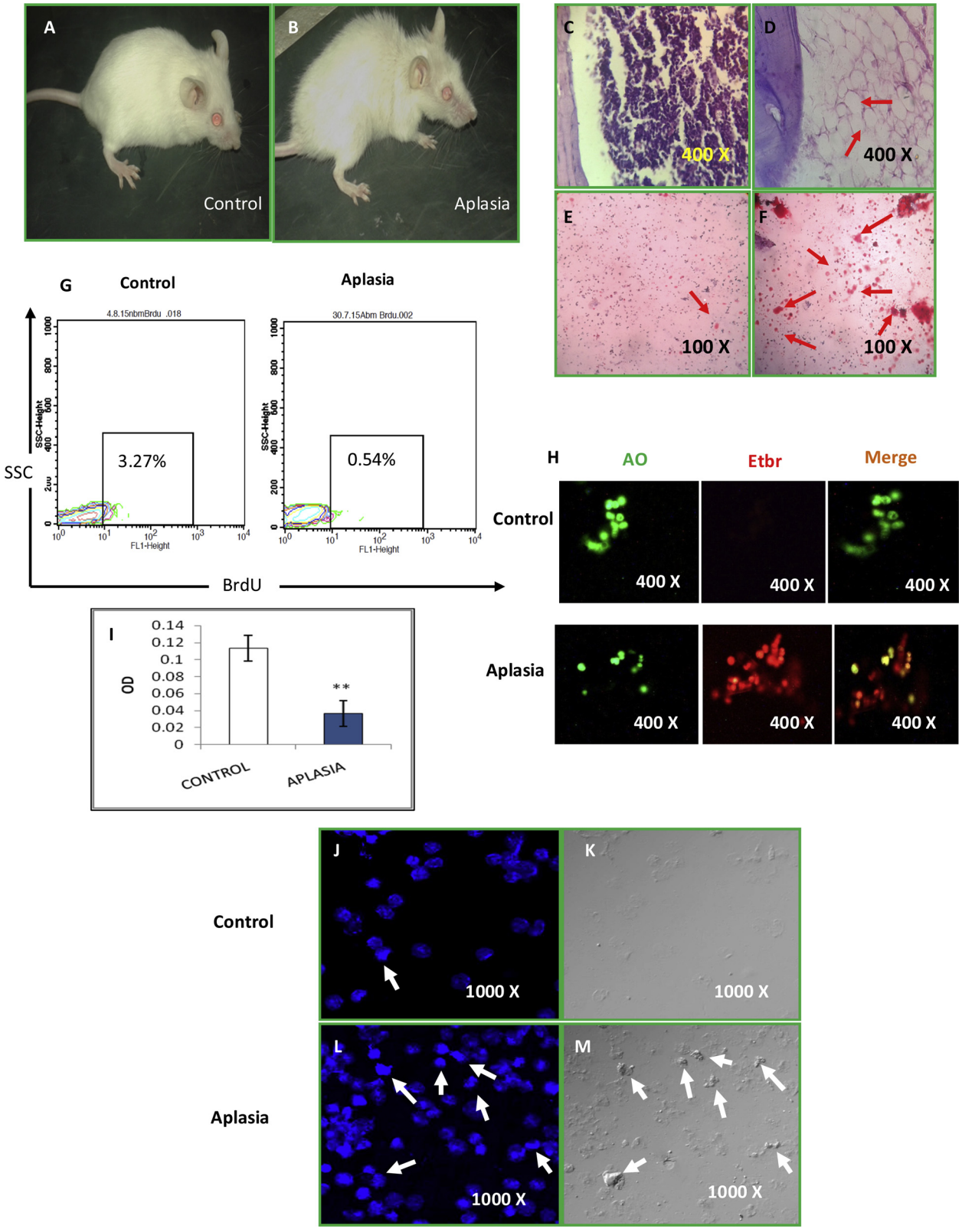
Cytotoxic effects of chemotherapeutic drugs on BM cells leading to aplastic anemia were determined by MTT assay. In brief, 200 µl of single cell suspension containing 2×10^6 BM cells from both the groups were incubated with 20 µl of MTT solution (5 mg/ml PBS) at 37 °C for 2 h in dark. After that, cell pellet was obtained and lysed with 100 µl DMSO. Finally, 2 ml of distilled water was added to the end products from both the groups and resultant OD was measured at 540 nm. Increase in OD due to formazan production was inversely related to the percentage of non-viable cells by drug`s cytotoxicity.

2.8. Apoptosis study

Apoptosis in marrow cells was determined by conventional acridine orange/ ethidium bromide (Ao/Etbr) fluorescent staining [45]. In brief, BM single cell suspension was mixed with dual fluorescent staining solution containing equal volume of 100 µg/ml AO and 100 µg/ml Etbr. Finally, the suspension was transferred to glass slides, covered with cover slip and immediately examined under fluorescent microscope (Axio Scope.A1, Zeiss). Etbr$^+$ cells were considered as apoptotic cells.

2.9. Nuclear chromatin study

Nuclear chromatin condensation and / or nuclear fragmentation are the hall mark for genomic damage and apoptosis [42,46–49]. Briefly, 1×10^6 BM cells from both the groups were transferred on glass slides and mounted with DAPI shield (Sigma Aldrich, USA). Finally, the slides were examined under laser scanning confocal microscope (FV1200, Olympus) and observations at minimum 20 random fields were taken into consideration.



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2.10. Determination of intracellular reactive oxygen species (ROS) generation

Intracellular ROS can be determined by using Dihydroethidine (DHE; Sigma Aldrich, USA), a substance which can be oxidized by superoxide anion to become Etbr, emiting red fluorescence [5,50]. BM cells at a density of 10^6 cells/ml were treated with 20 µl of 30 mM DHE (10 mg DHE in 1 ml DMSO) for 30 min at dark. The intensity of resultant red fluorescence was determined flowcytometrically (BD-FACS Calibur, Cell quest pro software; BD Bioscience, USA).

Fig. 1. Xenobiotic insults affect the mice by generating hematopoietic catastrophe. Healthy physical texture was found in control group of mice (A) where as feeble condition with prominent hunch back posture was noticed in case of drug affected mice (B). Histological study showed a normocellular composition of bone marrow in control group (C) but shrinkage of hematopoietic space together with increase of adipocytes was evident in aplastic marrow (D). Oil Red O staining also confirmed the excessive fat deposition (which took red colorations) (arrows) in aplastic marrow smears (F) in comparison with control marrow (E). Severe Replicative senescence of bone marrow due to aplastic stress was captured flowcytometrically by BrdU incorporation assay (G). A drastic decrease of BrdU incorporating cells was found in aplastic marrow in comparison to control. Acridine orange/ Ethidium bromide dual fluorescent staining (H) depicted the increased uptake of Etbr staining by aplastic marrow cells due to apoptotic nature. Severe cytotoxicity due to xenobiotic stress was identified behind the entire hematopoietic catastrophe as depicted by the decreased OD of resultant of MTT assay product (I).Confocal fluorescent imaging as well as phase contrast pictures showed chromatin abnormalities (arrow) in aplastic marrow cells (L, M) as compared to that of control (J, K). Values in bar represents mean ± SD and statistical significance represented by P values (student`s t test; **P < 0.01).

2.11. Assessment of mitochondrial membrane potential

Mitochondrial transmembrane electrochemical gradient ($\Delta\psi_m$) was determined by means of $Dioc_6(3)$ staining [51,52]. Briefly, 5 µl of 10 µM $Dioc_6(3)$ (Sigma Aldrich, USA) was added to ~ 10^6 cells suspended in 1 ml of tissue culture medium and incubated at 37° C for 20 min in dark. Cells were washed properly and analyzed with flowcytometry. Cell population with intact mitochondrial membrane potential emitted green fluorescence (FL-1 population) and those with collapsed mitochondrial membrane potential were negative for green fluorescence.

2.12. Flowcytometric analysis of protein expressions in basic hematopoietic population

BM cells were fixed with 1.5% PFA, permeabilized with 90% chilled methanol, washed thoroughly with PBS and finally suspended in FACS fluid. Cells from both the groups were divided into 8 sorting tubes each containing 2×10^6 BM cells. 2 µl of each antibodies against p21 (Cell Signaling Technology, USA), ATM (Cell Signaling Technology, USA), Chk-2 (Biolegend, USA), p53 (BD Bioscience,USA), Ndn (Santa Cruz Biotechnology, USA), Gfi-1 (Santa Cruz Biotechnology, USA), c-myc (Biolegend, USA), KU-80 (Cell Signaling Technology, USA), Sod-2 (Cell Signaling Technology, USA) were added and incubated at 37° C for 30 min. It was followed by the addition of respective secondary antibodies tagged with AlexaFluor-488 and incubation at 37° C for 30 min in dark. Finally, the expressional study of respective proteins was done in marrow cells (10,000 events) by flowcytometry. Histogram overlay analysis for the proteins was done at the low SSC and low FSC zone of scatterogram which was reported to be rich in Sca-1 positive hematopoietic stem/ progenitor cells (HSPCs) [5,32,42,44,53–56].

2.13. Statistical analysis

Quantitative data were represented as mean ± SD. Unpaired student`s *t*-test was performed for comparison of two groups (control and aplasia parameters) using GraphPad software (San Diego, USA). Statistical significance were set at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$. Each experiment was repeated for at least three independent times.

3. Results

3.1. Blood and BM cellularity

Hemogram values [Table 1] showed significant pancytopenia in mice with drug induced aplastic anemia as compared to control. Mean WBC (3.59 cells/mm^3 versus 8.43 cells/mm^3; $P < 0.001$), RBC (5.51 cells/mm^6 versus 7.98 cells/mm^6; $P < 0.01$), thrombocyte (214.36 cells/mm^3 versus 440.08 cells/mm^3; $P < 0.001$), hemoglobin (8.83 gm/dl versus 13.85 gm/dl; $P < 0.05$) and retic content (0.32% versus 1.47%; $P < 0.05$) were dramatically reduced in aplastic condition. Notable changes were also observed in differential WBC count. Severe neutropenia (8% ± 2% versus 25.67% ± 2.52%; $P < 0.001$) was found in aplastic mice indicating myeloablative potentials of BU and CP.

Aplastic group demonstrated shrinkage of hematopoietic space in BM and concomitant overshoot of non-hematopoietic adipocytes as evidenced by histological study [Fig.-1 C,D] and Oil Red O staining [Fig.-1 E,F].

3.2. Cytotoxicity and cell death in aplastic marrow

Xenobiotic stress during marrow aplasia conferred severe cytotoxic effects on cell viability. Mean OD of the resultant product of MTT assay reduced significantly in case of aplastic marrow (0.03 versus 0.11; $P < 0.05$) [Fig.-1 I]. Moreover, AO/ Etbr dual staining revealed the presence of huge number of apoptotic marrow cells during aplastic anemia identified by Etbr positivity [Fig.-1 H]. DPAI staining pattern as well as phase contrast pictures obtained from confocal microscopy [Fig.-1 J–M] depicted the scenario of nuclear chromatin condensation in marrow cells of aplastic mice which further reinforced the apoptotic effects of BU and CP.

3.3. Replicative senescence in marrow cells during aplastic anemia

BM cell proliferation was studied by BrdU incorporation (S-phase marker) assay. Cells taking thymidine analogue BrdU were considered to have undergone through S-phase and the cells stucked at G0/G1 phase of cell cycle were BrdU negative [57]. Cell obtained from the mice post 18 h of BrdU treatment showed significant decrease ($P < 0.01$) in the number of $BrdU^+$ cells in aplastic marrow (0.72% ± 0.06%) in comparison with control one (3.68% ± 0.87%) [Fig.-1 G]. The data revealed the loss of replicative efficacy of marrow cells under aplastic stress.

3.4. Accumulation of DNA injuries in marrow cells in aplastic condition

Accumulation of DNA damage is one of the common clues behind both mitotic catastrophe and cellular apoptosis [58–61]. As mentioned earlier, confocal microscopic observations of DAPI stained BM cells [Fig.-1 J-M], confirmed the presence of endogenous genomic insults in aplastic condition. Moreover AO/ Etbr dual staining can also be regarded as a useful tool to detect DNA injuries [45,62]. Increased uptake of Etbr by aplastic marrow [Fig.-1 H] cells further strengthened the genotoxic efficacy of the aplasia inducing drugs.

3.5. Maintenance of p21 level in aplastic marrow by DNA damage in p53 independent manner accounts for proliferative hindrance

P21 is a pan CDK-inhibitor that stalls cell cycle through its interaction with cyclin-CDK complex [63]. Though p21 is a common downstream molecule of p53, its activity is not always p53 dependent [64]. It has been reported earlier that persistence of constant cellular p21 level occurs due to the accumulation of endogenous DNA injuries as the latter inhibits ubiquitin-proteosome mediated degradation of p21 [65]. Elevated level of p21 was found in aplastic hematopoietic population (MFI; 127.92 ± 5.6 versus 97.02 ± 8.30; $P < 0.01$) and surprisingly the common p21 activator, p53 remained downregulated (MFI; 10.91 ± 0.52 versus 19.26 ± 1.99; $P < 0.01$) [Fig.-2 A, B, I]. To validate the p53 independent nature of p21 upregulation, we

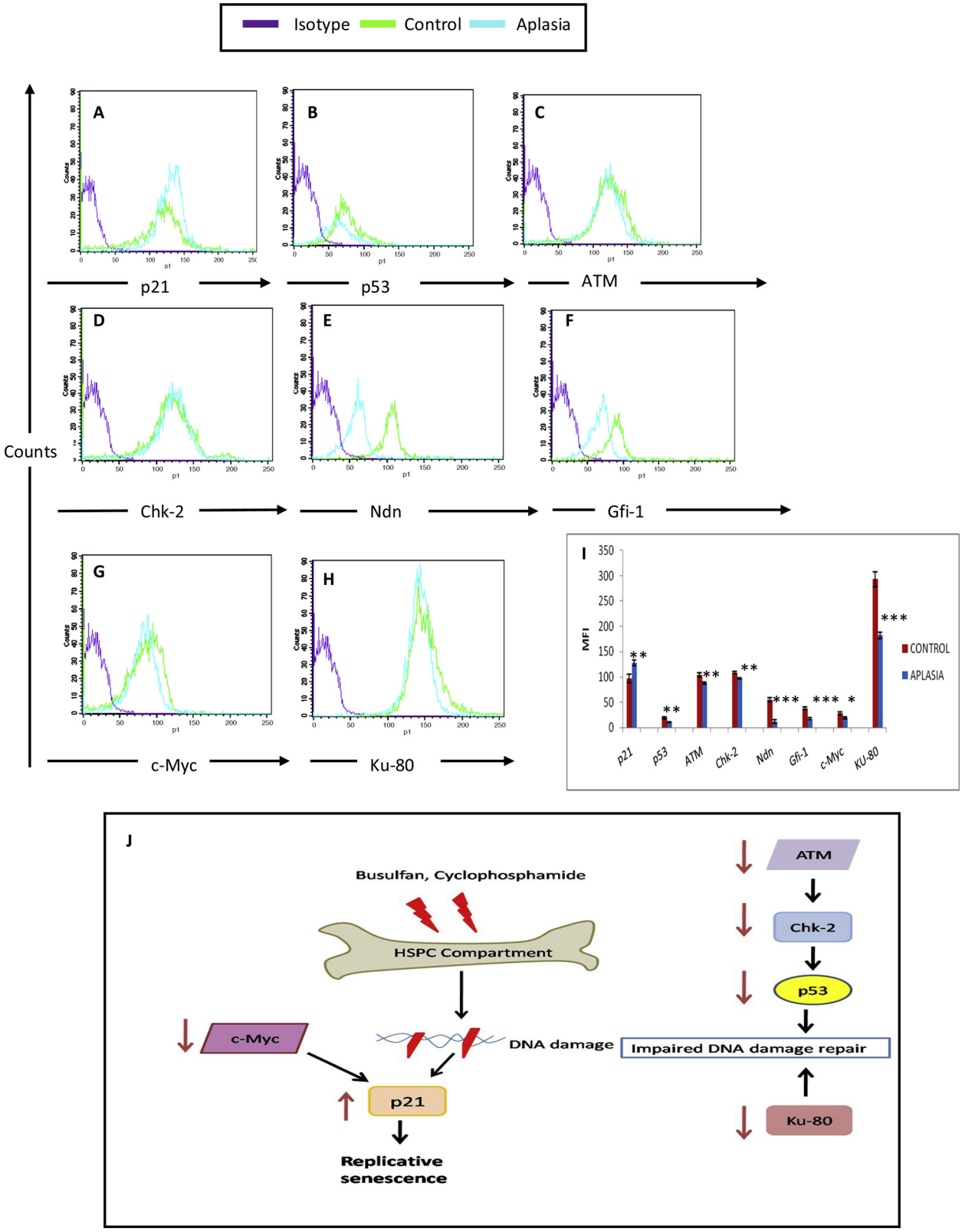

Fig. 2. Alteration of p21, p53 and related components in HSPC compartment under aplastic stress. Histogram overlay analysis depicted the increase of p21 (A) and decrease of p53 pathway components viz; p53, ATM, Chk-2, Ndn, Gfi-1 (B–F) in aplastic marrow HSPC compartment. c-Myc and Ku-80 (G, H) were also found to be decreased in aplastic condition. Panel-I represents the graphical representation of the MFI (mean fluorescent intensity) values of the mentioned protein components. Panel-J depicts the correlative association of the expressional alterations of the mentioned molecules with the replicative senescence and impaired genomic damage repairing mechanisms in HSPC compartment.
(P values; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Table 2
Mean fluorescence intensity (MFI) represents the expression pattern of different proteins.

Proteins	MFI of control (X ± SD)	MFI of aplasia (X ± SD)	P-value
p21	97.02 ± 8.30	127.92 ± 5.6	P = 0.0059
p53	19.26 ± 1.99	10.91 ± 0.52	P = 0.0022
ATM	103.94 ± 4.33	88.14 ± 1.89	P = 0.0044
Chk-2	108.7 ± 2.23	96.57 ± 1.3	P = 0.0013
Ndn	54.86 ± 3.92	11.60 ± 3.76	P = 0.0002
Gfi-1	38.05 ± 2.97	17.33 ± 1.81	P = 0.0005
C-myc	28.03 ± 2.36	19.2 ± 2.36	P = 0.0103
KU-80	293.18 ± 14.72	182.22 ± 6.67	P = 0.0003
Sod-2	280.87 ± 2.84	202.76 ± 3.12	P < 0.0001

examined the expressional levels of the upstream molecules of p53 activation pathway as well as sole p53 dependent downstream molecules important for hematopoietic physiology [53]. Interestingly, ATM, Chk-2 at the upstream of p53 as well as downstream cascade molecules Ndn and Gfi-1 were found to be significantly reduced in aplastic marrow HSPC compartment (MFI; ATM: 88.14 ± 1.89 versus 103.94 ± 4.33; P < 0.01, Chk-2: 96.57 ± 1.3 versus 108.7 ± 2.23; P < 0.01, Ndn: 11.60 ± 3.76 versus 54.86 ± 3.92; P < 0.001, Gfi: 17.33 ± 1.81 versus 38.05 ± 2.79; P < 0.001) indicating the collapse of p53 pathway during aplastic anemia [Fig.-2 C–F, I]. The findings [Table-2] also hinted towards the p53 independent activation of p21 under aplastic stress [Fig.-2 J]. Downregulation of c-myc oncogene is often found to be associated with the p53 independent p21 upregulation [66,67]. Our observation also revealed the significant (P < 0.05) decline of c-myc in aplastic HSPCs (MFI; 19.2 ± 2.36) in comparison with control (28.03 ± 2.36) [Fig.-2 G, I].

3.6. Generation of redox imbalance in aplastic marrow

DNA damage often leads to production of free radicals in cell causing redox imbalance [68,69]. An increment in the production of ROS in aplastic marrow was detected by DHE staining. DHE is a non-fluorescent lipophilic compound which in presence of ROS gets converted into fluorescent Ethidium [52]. Intensity of the fluorescent produced due to ethidium formation can correlate with the amount of ROS produced [70]. Flowcytometric study [Fig.-3 A, B] revealed a significant rise in ROS level in marrow under aplastic stress (MFI; 425.36 ± 12.62 versus 117.74 ± 9.71; P < 0.0001).

3.7. Depolarization of mitochondrial transmembrane potential causes apoptosis in aplastic marrow

Intracellular ROS production interferes with mitochondrial activity by inducing transmembrane potential loss leading to the activation of intrinsic apoptotic pathway [71–73]. In this study we used carbocyanine dye $Dioc_6(3)$, a lipophilic cell permeable flurochrome, to determine mitochondrial membrane potential (MTP) [74]. Mitochondrial uptake of $Dioc_6(3)$ is dependent on MTP ($\Delta\psi_m$). A positive correlation was found between the ROS production and collapse of $\Delta\psi_m$ in aplastic marrow cells. A significant increase in the percentage of $\Delta\psi_m^{low}$ cells (27.90% ± 2.21 versus 10.19 ± 1.42; P < 0.001) [Fig.-3 C–E] was concomitant with the elevation of ROS level which might imply towards the apoptotic elimination of hematopoietic cells under the influence of BU and CP [Fig.-3 G].

3.8. Irreversible marrow damage by BU and CP

Onset of premature senescence and apoptosis in drug affected marrow occurred due to the direct consequences of DNA damage accumulation. In this regard, downregulation of p53 cascade seemed to have immense significance. Though like p21, cytostatic influence is exerted by p53 but the pathway accounts for the repairing of DNA damage and thereafter cellular re-entry to cell cycle [75,76]. Thus downregulation of p53 added to the cellular devastation due to the impairment of corrective measures [Fig.-2 J]. KU-80 is another important player for DNA break repair by non-homologous end joining [77]. Downregulation of KU-80 is associated with increased accumulation of DNA damage leading to premature aging [78–80]. Significant decrease in KU-80 level during bone marrow aplasia (MFI; 182.22 ± 6.67 versus 293.18 ± 14.72; P < 0.001) [Fig.-2 H, I] reinforced the irreversibility of the genotoxic damage [Fig.-2 J]. Moreover, the aplastic cells suffered through the significant decline of anti oxidant enzyme Sod-2 (MFI; 202.76 ± 3.12 versus 280.87 ± 2.84; P < 0.0001) [Fig.-3 F] which indicated towards the irreversibility of the oxidative damage too [81,82] [Fig.-3 G].

4. Discussion

Drugs like BU, CP are widely used for chemotherapeutic purposes but often exert dreadful side effects on hematopoietic system leading to marrow failure condition referred as aplastic anemia. However, the precise molecular mechanisms by which the agents affect BM still remained unclear. We have reported in our previous study that BU and CP impairs hematopoietic homeostasis in two ways: firstly they induce mitotic catastrophe in hematopoietic cells and secondly trigger cellular apoptosis [30,32,42]. Our present results of BrdU incorporation assay, cytotoxicity assay with MTT as well as AO/Etbr staining also depicted the collapse of hematopoiesis due to stalling of cell division together with increased apoptotic elimination of BM cells.

To gain insight into the molecular mechanisms underlying replicative senescence and apoptosis in aplastic marrow, we evaluated the extent of genotoxic insult caused by BU and CP. Increased chromatin condensation and fragmentation in BM cells as depicted by DAPI staining confirmed the generation of nuclear damage by aplastic stress. Moreover, cellular susceptibility to Etbr staining reinforced the accumulation of extensive DNA injuries in aplastic condition.

Common DNA damage response includes elevation of cell cycle inhibitors, induction of redox imbalance, alteration of mitochondrial membrane potential etc. [65,68,69,83]. The present study documented a significant rise in cell cycle inhibitor p21 in HSPC compartment during marrow aplasia which can be a manifestation of the DNA damage that ultimately accounts for the replicative senescence. Though p21 is a common downstream player of p53 but in aplastic condition its activation seemed to be p53 independent. All the players of p53 cascade viz; ATM, Chk-2, p53 as well as sole p53 dependent hematopoiesis regulators Ndn and Gfi-1 got significantly downregulated during bone marrow aplasia. Together with the upregulation of p21, c-myc was found to be significantly decreased in aplastic marrow cells. This finding is in agreement with previous study showing p53 independent activation of p21 involves c-myc downregulation.

Downregulation of p53 pathway in aplastic marrow may certainly have immense significance. P53 allows repairing of cellular damage and promotes re-entry into cell cycle [75,76,84]. Thus, p53 downregulation in aplastic marrow hinted towards irreparable nature of genotoxic insults by BU and CP. Downregulation of another DNA repairing protein KU-80 also added to the irreversibility of genomic damage in aplastic condition.

Apart from being responsible for the replicative senescence, DNA damage was also found to increase free radicals in aplastic marrow. DHE staining confirmed the redox imbalance generated by BU and CP owing to the elevation of ROS production. ROS has a vice-versa effect with DNA damage. Moreover, it may account for cellular apoptosis by disrupting mitochondrial physiology. Simultaneous elevation of ROS and loss of mitochondrial transmembrane potential in aplastic marrow as depicted by $Dioc_6(3)$ staining pointed towards the ROS mediated triggering of mitochondrial apoptotic pathway due to DNA damaging effects of BU and CP. Downregulation of antioxidant enzyme Sod-2

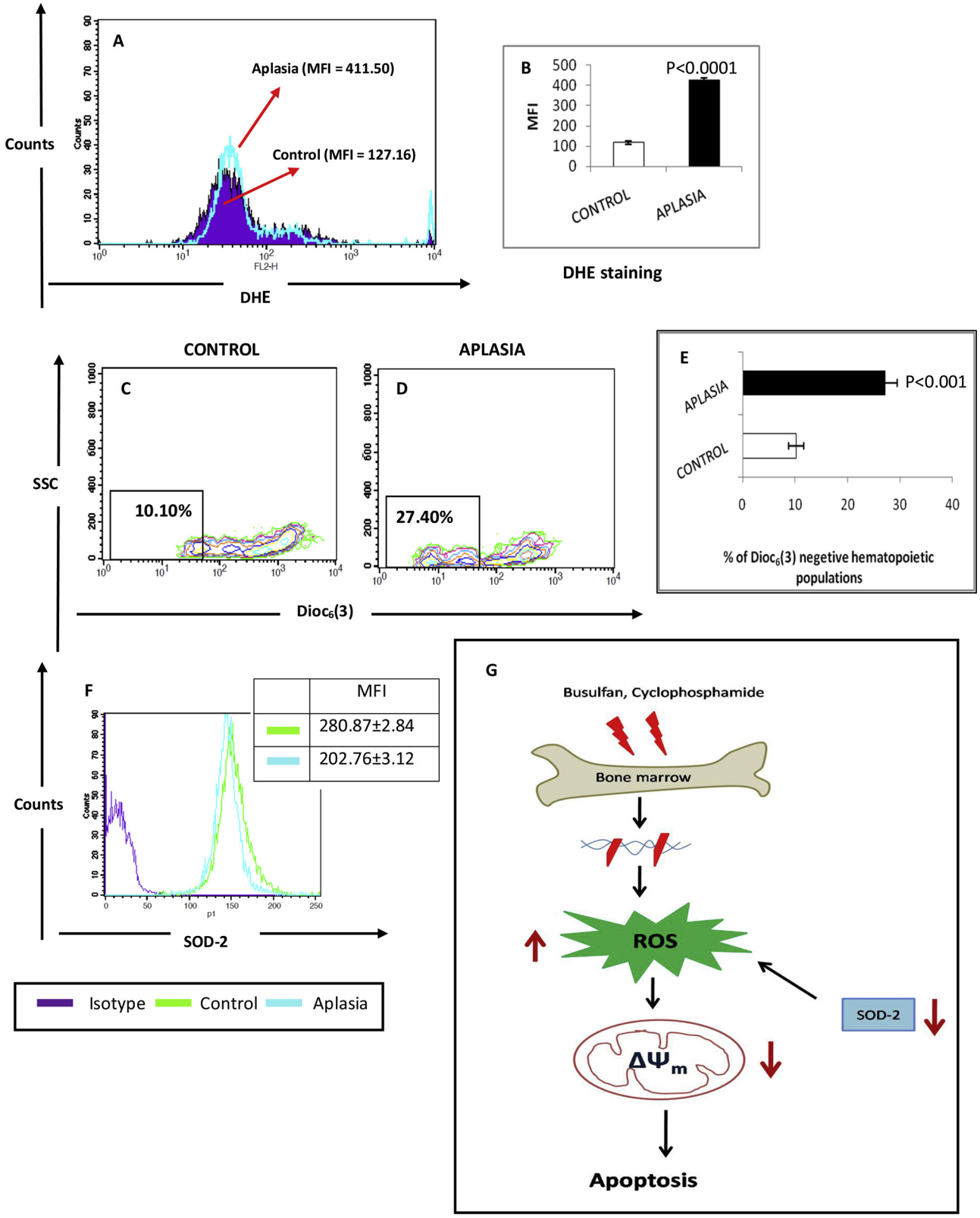

Fig. 3. Severe redox disbalance leading to mitochondrial transmembrane potential loss in aplastic marrow. Flowcytometric histogram overlay analysis (A, B) showed significant increase in the generation of ROS in aplastic as compared to that of control. $Dioc_6(3)$ staining showed that the mitochondrial transmembrane potential ($\Delta\psi_m$) was also found to be significantly decreased in aplastic marrow cells (D, E) as compared to that of control (C, E). Flowcytometric analysis also depicted expressional decline of SOD-2 in aplastic marrow (F). Panel-G represents the correlative association of redox imbalance and collapsed mitochondrial membrane potential leading to the cellular apoptosis in marrow compartment due to aplastic stress.

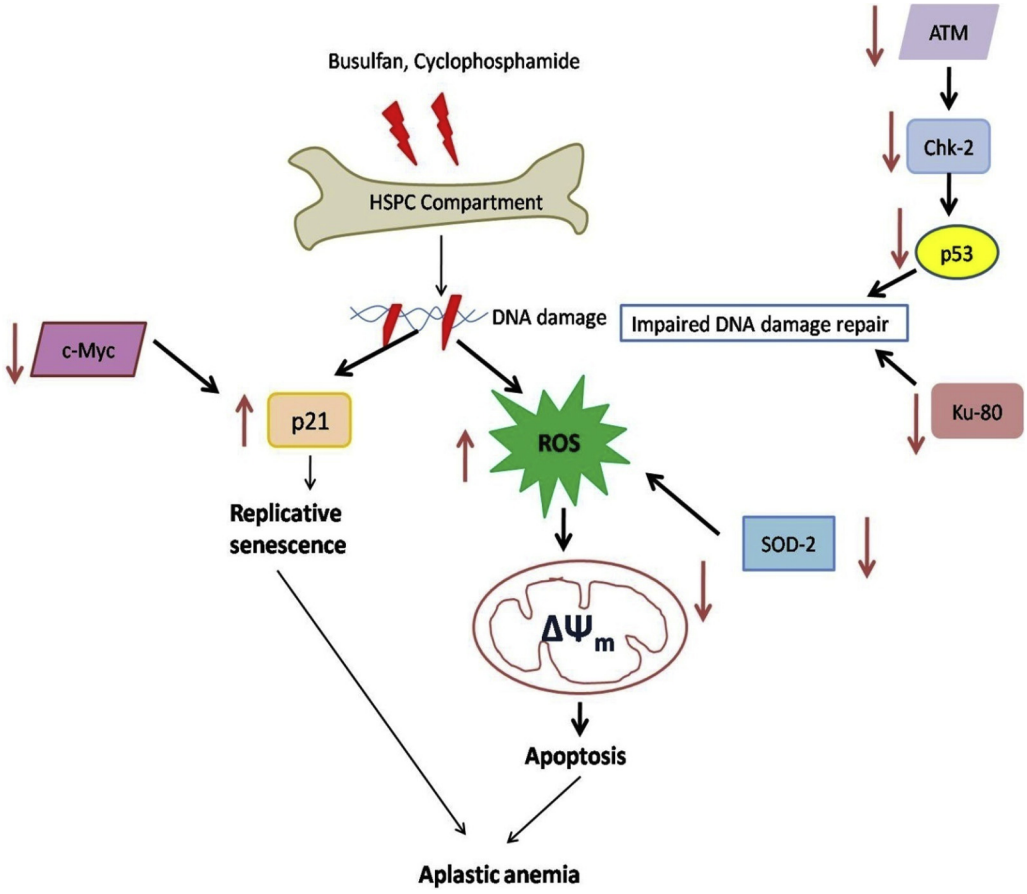

Fig. 4. Schematic representation of the study outcome. Chemotherapeutic drug administration resulted in the accumulation of DNA damage in the bone marrow that caused p53 independent upregulation of p21 leading to replicative senescence. On the other hand DNA damage induced ROS production lead to mitochondrial membrane potential collapse resulting in cellular apoptosis. Downregulation of entire p53 pathway, decline of KU-80 level were responsible for the irreparable nature of the DNA damage. Decreased SOD-2 impaired the repairing of the oxidative damage. Entire signaling deregulation resulted in the transformation of normal marrow physiology to aplastic pathophysiology.

hinted towards the impairment of oxidative dagage repairmen system under aplastic stress.

Taken together all the above findings, it can be concluded that the genotoxic effects of BU and CP induces p21 mediated replicative senescence and ROS mediated cellular apoptosis in marrow HSPCs leading to aplastic pathophysiologies. The knowledge about the network [Fig.-4] behind the ablation of marrow cells during drug induced aplastic anemia may certainly potentiate the designing of newer cytotherapeutic approaches critically needed for the treatment of the dreadful condition.

Conflict of interest

All authors declared no potential conflict of interest.

Acknowledgements

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