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Scientific paper

ALTERED CANONICAL HEDGEHOG-GLI SIGNALLING AXIS IN PESTICIDE-INDUCED BONE MARROW APLASIA MOUSE MODEL

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The mechanistic interplay between pesticide exposure and development of marrow aplasia is not yet well established but there are indices that chronic pesticide exposure in some instances causes marrow aplasia like haematopoietic degenerative condition in human beings. Canonical Hedgehog (Hh) signalling has multiple roles in a wide range of developmental processes, including haematopoiesis. The present study was designed to explore the status of four important components of the canonical Hedgehog signalling cascade, the Sonic Hedgehog (Shh), Ptch1, Smo, and Gli1, in a mouse model of chronic pesticide-induced bone marrow aplasia. We used 5 % aqueous mixture of pesticides (chlorpyriphos, prophenophos, cypermethrin, alpha-methrin, and hexaconazole) for inhalation and dermal exposure of 6 hours per day and 5 days a week up to 90 days. Murine bone marrow aplasia related to chronic pesticide treatment was confirmed primarily by haemogram, bone marrow cellularity, short term bone marrow explant culture for cellular kinetics, bone marrow smear, and flow cytometric $Lin^{-}Sca\text{-}1^{+}C\text{-}kit^{+}$ extracellular receptor expression pattern. Later, components of hedgehog signalling were analysed in the bone marrow of both control and pesticide-treated aplastic groups of animals. The results depicted pancytopenic feature of peripheral blood, developmental anomaly of neutrophils, depression of primitive stem and progenitor population along with Shh, Ptch1, Smo and Gli1 expression in aplasia group. This investigation suggests that pesticide-induced downregulation of two critically important proteins - Ptch1 and Gli1 - inside the haematopoietic stem and progenitor cell population impairs haematopoietic homeostasis and regeneration mechanism *in vivo* concurrent with bone marrow aplasia.

KEY WORDS: *bone marrow suppression, haematopoietic stem cell, patched, smoothened, sonic hedgehog*

A surge of numerous organophosphates, pyrethroids (analogs of naturally occurring phytopyrethrins), and thiazole fungicides replaced DDT after the ban in 1993. Depending on the physiological activity, synthetic pyrethroids are of two distinct types: Type I pyrethroids, which do not contain the α-cyano group, and Type II pyrethroids, which have the α-cyano group (1-3). Previous studies have affirmed the genotoxic and/or cytotoxic effects of Type II pyrethroids such as cyfluthrin, cypermethrin etc. (4-9). The carcinogenic potential of pyrethroids was also reported on the murine model (9). A few recent studies revealed that besides blocking neurotransmission, both pyrethroids and organophosphate pesticides can cause

immunosuppression and deregulation of many signalling molecules (10-15). Organophosphates (derivatives of phosphonic acid) and systemic azole fungicides have profound cytotoxic activities, too (16, 17). Despite a few epidemiological reports (18-20), we thus far have meagre understanding of the haematopoietic failure and the development of bone marrow aplasia by pesticide toxicity. Bone marrow aplasia represents a rare disorder of haematopoietic stem cell failure, often with severe life-threatening pathological consequences. More than half of the diagnosed cases of the aplastic anaemia are idiopathic, rest being acquired or inherited. Toxic chemicals, radiation, viral infections, and many other environmental factors are associated with frequent occurrence of acquired aplastic anaemia (18, 21-26). Acquired bone marrow aplasia may arise due to one of the three events: (i) intrinsic stem cell defect due to cytotoxic chemicals, (ii) disturbed stem cell microenvironment or (ii) deregulated stem cell differentiation process. Our previous studies have confirmed stem cell depletion in the pesticide exposed mouse model of marrow aplasia and in aplastic patients with history of chronic pesticide exposure (27-29).

The present study was planned to explore the probable mechanism of bone marrow aplasia development induced by chronic pesticide toxicity in mouse involving Hedgehog (Hh) signalling. Hedgehog-Gli signalling axis is an important cytosolic regulator that controls many fundamental processes from embryonic life to adulthood. Members of hedgehog family proteins are the key entities in growth patterning and morphogenesis of different regions within the body plan, from Drosophila to human beings. Intracellular Hedgehog signal transduction is executed through the binding of Hedgehog ligands to the transporter-like receptor, a tumour suppressor protein Patched 1 (Ptch1), which releases an inhibitory effect of its own on Smoothened (Smo), a seven pass transmembrane protein, and ultimately the signal goes down to the nucleus by Gli transcription factors thereby activating Hedgehog targeted genes (30-35). Besides different activities, Hedgehog-Gli signalling axis controls primitive and definitive haematopoiesis during embryogenesis and postnatal condition (36-39). In contrast, two contemporary articles reported that hedgehog signalling was dispensable for definitive haematopoiesis in conditionally deleted $Smo^{-/-}$ in mice (40-41). But, in support of Hh and definitive haematopoiesis, Trowbridge et al. (44) demonstrated that constitutively Hh activated $Ptch^{-/+}$ in mice showed increased cycling and expansion of haematopoietic stem cells (HSC) under homeostatic conditions. On the other hand, Dierks et al. (45) showed that $Smo^{-/-}$ in murine cells lost the colony forming ability at the second replating time, in contrast to the $Ptch^{-/+}$ in murine cells where Smo was activated, which showed both regeneration and enhanced engraftment ability of the bone marrow result.

The involvement of Hedgehog signalling in definitive haematopoiesis ranges from normal haematopoiesis to differentiation and malignancy and these have been supported by numerous investigators (46-51). Hedgehog activity specifies cell fate either through long-range or short-range individual signalling and has also been associated with proliferative responses of target cells (31). Expression of Hedgehog in human haematopoietic population and cells comprising haematopoietic microenvironment suggests that Hedgehog proteins play a functional role in blood cells. Blood cells and haematopoietic microenvironment transduce Hedgehog signalling which has downstream Gli involvement (47). Therefore, the studies on self-renewal and expansion of HSC from bone marrow in pesticide-induced aplastic animal model were taken into consideration as an innovative experimental strategy to reveal the mechanism that lies beneath the Hh-Gli signalling pathway. In corroboration of the aforesaid studies related to hedgehog signalling and definitive haematopoiesis, we are the first group to suggest that chronic long-term pesticide exposure downregulate Hh-Gli signalling axis, which accelerates bone marrow failure or aplasia in a mouse model.

MATERIALS AND METHODS

Animals

Ten to twelve-week-old Swiss albino mice of both sexes (20 males and 20 females) for pesticide exposure purpose and 10 animals (5 males and 5 females) for control weighing 20 g to 24 g were selected from an inbred colony maintained under controlled room temperature (22±2) °C in the animal house of the Calcutta School of Tropical Medicine (India). Both male and female animals were equally used in all following assays. The animals were fed a standard recommended diet and water *ad libitum*, under

standard conditions with a 12-hour light-dark period. Throughout the experiment, a maximum of six animals were housed in one cage containing sterile paddy husk as bedding. The procedures followed were in agreement with the approved guide for the care and use of laboratory animals, the Institutional Animal Ethical Committee (IAEC), and EU Directive 2010/63/EU.

Pesticide mixture preparation

In India, farmers and others use pesticides in a mixture and without any self-protection, so, we took a mixture of pesticides for the experimental purpose to mimic the normal exposure of individuals. We also observed and published the immunohaemotoxic effects of pesticide exposure where patients with aplastic anaemia, mainly farmers, used mixed pesticides (29). We used a 5 % aqueous mixture of DURSBAN (Chlorpyriphos 20 %; Dow Agrosciences, India), PROFEX SUPER (Prophenophos 40 % + Cypermethrin 4 %; Nagarjun Agrochemicals Ltd. India), STOP (alpha-Methrin 10 %; Biostadt, India), and Hexaconazole (Sigma-Aldrich, USA). The first three easily available commercial pesticide preparations were of organophosphate and pyrethroid groups and were manufactured by local Indian agrochemical companies as mentioned above. All aforementioned chemicals were dissolved in aromatic hydrocarbon solvent as per information provided by the manufacturer on each pesticide container. The last one was a systemic fungicide rarely available in the local market as HEXACON SUPER (Hexaconazole 5.43 %). Due to its (HEXACON SUPER) paucity, we used Hexaconazole from Sigma-Aldrich, USA, in pure form and maintained the same concentration as in HEXACON SUPER.

Pesticide exposure

Adult mice (10 to 12 weeks old) were divided into two groups. Experimental animal group (N=40) received inhalation and dermal exposure [by handheld glass atomiser (local made), atomising time was 5 min] to 10 mL of 5 % aqueous solution of aforesaid pesticides mixture for 6 hours per day and 5 days a week up to 90 days (LD_{50} of 10 % aqueous solution was found to be 20 days in our previous experiments). Control group (N=10) received inhalation and dermal exposure of aqueous saline solution without any trace of pesticide contamination over the same period of time (28). Altogether we took 24 exposed animals (8 animals per experiment) and 6 control animals (2 animals per experiment) for the following flow cytometric experiments which were repeated three times along with bone marrow cellularity assessment. The rest of the exposed animals (N=16) and control animals (N=4) were used for the bone marrow smear analysis and explant culture experiments. However, peripheral blood haemogram experiment was done for each and every animal as the investigation was related to the degeneration of haematopoietic machinery. Each haemogram experiment was performed with randomly selected five control animals and twenty pesticide-treated animals at a time.

Blood haemogram profile

We randomly selected experimentally pesticide-treated (N=20) and control (N=5) animals from respective cages at a time for blood haemogram profiling after completing 90-day exposure. Approximately 200 µL of blood was collected in heparinised vial by tail vein puncture from the animals. Total erythrocyte (RBC) count, total leukocyte (WBC) count, platelets, reticulocytes, differential WBC count, and haemoglobin estimation were performed manually as per standard laboratory protocol. Total RBC, WBC, and platelet count were performed by haemocytometer (Rohem, India) and binocular light microscope (Olympus *Ch20i*). Reticulocyte count was performed by Brilliant Cresyl Blue (Sigma) staining method and haemoglobin concentration was estimated by colorimetric (Systronics, India) cyanomethaemoglobin method. At the end, differential WBC count was done by Leishman staining (Himedia, India).

Bone marrow isolation and single cell preparation

The chronic pesticide-exposed experimental and control groups of mice were sacrificed to isolate the long bones (femur and tibia). Bone marrow was flushed out from the isolated bones by syringe (Dispovan 2 mL) containing RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10 % FBS (Foetal Bovine Serum, Lonza, South American origin). Following bone marrow collection, a part of it was kept as undisturbed for explant culture and bone marrow smear analysis. Other part was subjected to single cell preparation for the assays such as total cellularity assessment, quantification of bone marrow-derived stem/progenitor cells, and status of hedgehog signalling in the bone marrow haematopoietic cells.

Bone marrow smears analysis

Bone marrows of the normal and exposed groups were Leishman stained (Himedia; India) and examined under light microscopy (1000x magnification; Olympus *Ch20i*) (52).

Bone marrow cellularity assessment

Femoral marrow cells were aseptically collected by flushing. Total cell counts of marrow single cell suspensions of both control and pesticide-exposed animals were then performed in Hemocytometer chamber (Rohem INDIA) by well known Erythrosin-B (Sigma-Aldrich, USA) dye exclusion method (53).

Bone marrow explants culture and cell release pattern study at (0, 24, 48, 72, 96) hours

Small portions of the isolated (described previously) marrow were cut into small pieces (0.2 mm^3) and subjected to culture in triplicate (for each of the normal and experimental groups) in 75 mm culture dish (Corning, USA) containing 3 mL of RPMI-1640 supplemented with 15 % FBS (Lonza, South American origin). The cultures were incubated at 37°C inside CO_2 incubator and a time-course [(0, 24, 48, 72, 96) h] cell release pattern was monitored and calculated by the aid of haemocytometer (Rohem; India).

Flow cytometry

Evaluation of murine bone marrow stem and progenitor population

To analyse haematopoietic stem cell population and signalling, $1x10^6$ pooled cells from each group were fixed with 1.5 % para-formaldehyde (PFA, Ranbaxy, India) for 30 min at 37 °C in dark. To identify the murine haematopoietic progenitors, a PerCP-Cy5.5™ conjugated Lineage (Lin) antibody cocktail (BD Pharmingen, USA) was used (CD3e, CD11b, CD45R/B220, TER-119, and Ly-6G and Ly-6C) in RBC depleted population for 20 min. This was followed by labelling with anti-mouse Sca-1-PE monoclonal antibody (BD-Bioscience, USA) and anti-CD117 antibody-FITC (BD-Bioscience, USA) in 1 % bovine serum albumin (Sigma), and dissolution in phosphate buffered saline (PBS) for another 30 min incubation. Furthermore, a single rapid wash was performed by PBS to remove unbound antibody from each sample. Samples were then analysed by BD-FACS Callibur (Becton Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA).

Flow cytometric analysis of Shh, Ptch1, Smo, and Gli1: components of canonical Hedgehog signalling pathway

Shh, Ptc1, and Gli1 proteins were stained by cell permeabilisation technique (27) in which $3x10^6$ bone marrow haematopoietic cells were first fixed at room temperature in 1.5 % PFA and pelleted. Fixed cells were permeabilised by resuspension with vortexing in 500 µL of chilled 90 % methanol (SRL, India.) per $1.5x10^6$ cells and incubated at 4 °C for 15 min to 20 min. Thereafter, cells were washed twice in FACS fluid (PBS containing 1 % BSA) and divided in five sorting tubes with $1.5x10^6$ cells per 100 µL of fresh FACS fluid. 2 µL anti-Shh antibody (Santa Cruz Biotechnology, USA), Ptc1antibody (H-267) (Santa Cruz Biotechnology, USA), and anti Gli1 antibody (H-300) (Santa Cruz Biotechnology, USA) were then added into respective sorting tubes and incubated for 30 min at 37 °C. This was followed by the addition of goat anti-rabbit secondary antibody conjugated with AlexaFluor-488 (Invitrogen, USA) to each primary antibody containing tubes and incubated further for 30 min at 37 °C.

One part of the non-permeabilised PFA-fixed bone marrow haematopoietic cells (1×10^6 cells) were subjected to 2 µL of anti-Smo antibody (N-19) (Santa Cruz Biotechnology, USA), which was directed against extracellular part of Smo receptor and incubated for another 30 min. This was followed by rabbit anti-goat secondary antibody conjugated with FITC (Santa Cruz Biotechnology, USA) staining. Then, all the samples were washed to remove excess fluorescence and resuspended to staining media. They were analysed by BD FACS Calibur (Becton-Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA).

Statistical analysis

All the values of flow cytometric studies, cell release kinetics, and haemogram were represented as mean ± SD (Standard Deviation). Statistical analysis was performed by paired Student's *t*-test and the level of statistical significance was $P<0.05$ and $P<0.0001$.

RESULTS

Peripheral blood pancytopenia

Comparative haemogram study in control and chronic pesticide-exposed group of mice revealed that

the pesticide-exposed animal group had moderate to severe pancytopenia with depressed haemoglobin level (~7 g dL^{-1}) and uniformly reduced corpuscular counts, of which reticulocyte count was significantly lower (~0.23 %) throughout the study compared to normal mice (0.89 % to 1.5 %). Significantly low absolute neutrophil count (498 μL^{-1}), appearance of abnormal neutrophils with their distorted nuclear lobulation pattern, and monocytopoenia (≤48 μL^{-1}) have further been recorded. The deserted situation of peripheral blood signified the commencement of bone marrow aplasia only inside these experimental animals as compared to the control group, which had normal total WBC (~6.0x10^3 μL^{-1}), RBC (8.5x10^6 μL^{-1}), and standard lymphocyte (~4320 μL^{-1}), neutrophil (~1420 μL^{-1}), and monocyte (~220 μL^{-1}) counts (Table 1).

Bone marrow smear study

Bone marrow smear of control group of mice revealed normal haematopoietic cellular distribution (Figure 1a). On the other hand, pesticide-exposed bone marrow cell pool started to be replaced by stromal fibres, large adipocytes, and empty spaces in contrast to the normal bone marrow (Figures 1b - 1d).

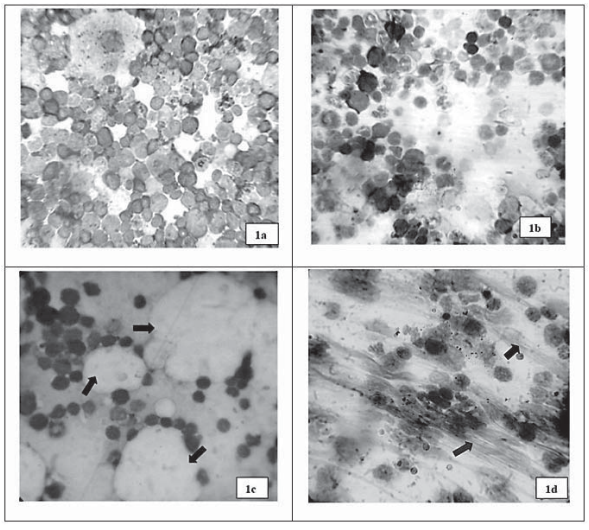

Figure 1 *Light microscopic study of bone marrow smear*
Figure 1a shows the smear of a control bone marrow comprised of numerous haematopoietic cells at different stages of developmental cascade. In the pesticide-treated aplastic bone marrow (Figures 1b-1d), most of the spaces in smear are occupied by large adipocytes or stromal fibres (arrow marked), which developed from pre-adipocytic fibroblasts and collagen, respectively, and very few haematopoietic cells are present there.

Table 1 *Comparative haemogram of control and pesticide-treated mice.*

Parameter	Control (N=10) Mean ±SD	Treated (N=40) Mean ±SD
Haemoglobin / g dL^{-1}	15.9±2.20	7.0±1.50 #
Reticulocyte / %	0.9±0.15	0.23±0.07 #
Total count		
Total RBC / x 10^6 μL^{-1}	8.5±0.70	3.2±0.60 #
Total WBC / x 10^3 μL^{-1}	6.0±1.20	3.6±0.30 #
Platelets / x 10^3 μL^{-1}	440±13.92	169±10.00 #
Differential WBC count		
PMNs / μL^{-1}	1402±2.00	498.8±3.12 #
Lymphocytes / μL^{-1}	4320±2.15	3045±3.60 #
Monocytes / μL^{-1}	220±1.30	48±1.50 #
Basophiles / μL^{-1}	58±0.01	10±0.05 #

 # Marked values are significantly different (P<0.001) from the values of the control animals within the same row.

Total bone marrow cellularity

Chronic pesticide exposure diminished (~2.5 fold) the total bone marrow cellularity of the pesticide-treated animals in comparison to the control group of animals. The difference in the bone marrow cellularity between control and pesticide-treated animals was statistically significant (P<0.05) (Figure 2).

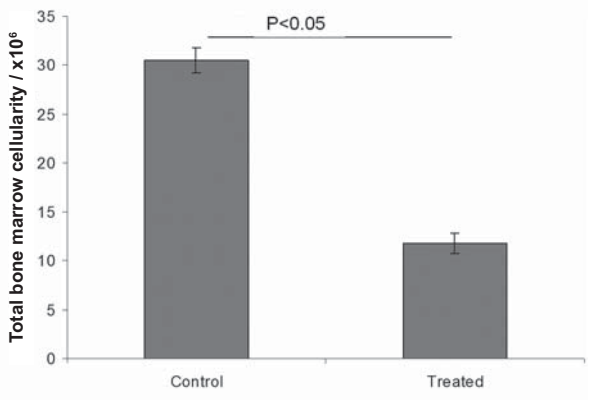

Figure 2 *Total bone marrow cellularity study*
Comparative whole bone marrow assessment of control and chronic pesticide-treated group of animals revealed a significant difference in its cellularity. Chronic pesticide exposure reduced the bone marrow cellular components approximately 2.5-fold in comparison to control group and observed difference was statistically verified (P<0.05).

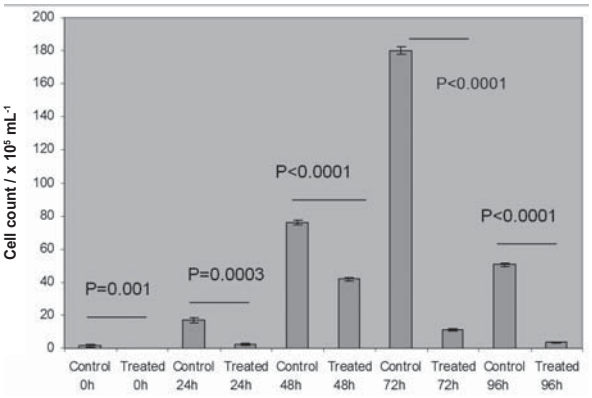

Figure 3 *Bone marrow cell release kinetics*

Bone marrow explant culture and cell release kinetics further revealed the hypocellularity of bone marrow after chronic pesticide exposure

Initially (0 h), normal bone marrow explant started to release cells and the number of cells gradually increased with time. The number of released cells was the highest at 72 h [(180±2.0) x10^5 cells mL^{-1}] in contrast to 24 h [(16.63±1.5) x10^5 cells mL^{-1}], 48 h [(76±1.3) x10^5 cells mL^{-1}], and 96 h [(50.6 ± 1.1) x10^5 cells mL^{-1}] (Figures 3 and 4). However, the scenario of aplastic bone marrow explant was not same (Figures 3 and 4). At the beginning (0 h) and after 24 h of culture, very few cells were released from pesticide-exposed bone marrow and it was almost negligible in comparison to normal cell release kinetics at 24 h (P=0.0003). But, the accelerated cell release was observed at 48 h [(41.76±1.32) x10^5 cells mL^{-1}] and was considered significant (P<0.0001). Cell release kinetics of aplastic bone marrow again decreased after 72 h [(11±0.5) x10^5 cells mL^{-1}] and 96 h [(3.5±0.2) x 10^5 cells mL^{-1}] (P<0.0001).

Hypocellularity was due to the shortage of stem/ progenitor cells in the bone marrow

The percentage of gated $Lin^-Sca\text{-}1^+C\text{-}kt^+$ (LSK) cells, (0.47±0.05) % [i.e. (0.01±0.002) % of whole bone marrow] in the bone marrow, showed a nearly sevenfold decrease in the pesticide exposed animals (P<0.001) compared to control animals, (3.42±0.7) % [i.e. (0.07±0.05) % of whole bone marrow] (Figure 5). It signifies that chronic pesticide exposures severely hampered the primitive haematopoietic stem cell compartment and its effects were manifested through hypocellular bone marrow and pancytopoenia in the treated group of animals.

Pesticide-mediated deregulation of canonical Hedgehog signalling: latent cause of bone marrow hypocellularity and degeneration

There was a severe depression in the expression of Ptch1 (Mean Fluorescence Intensity, MFI=320.4 ± 1.90), Smo (MFI=40.32±2.01), and Gli1 (MFI=365.89 ±3.45) proteins in the pesticide-treated bone marrow aplasia condition in comparison with the control bone marrow (MFI of Ptch1=400.0±3.2, MFI of Smo=75.85±1.65 and MFI of Gli1=550±3.54). Furthermore, bone marrow of control animals showed a good amount of autocrine Shh ligand expression (MFI=178.20±2.7) in contrast to the pesticide-treated bone marrow where Shh expression (MFI=46.35±3.70) was hampered. All the data were statistically significant

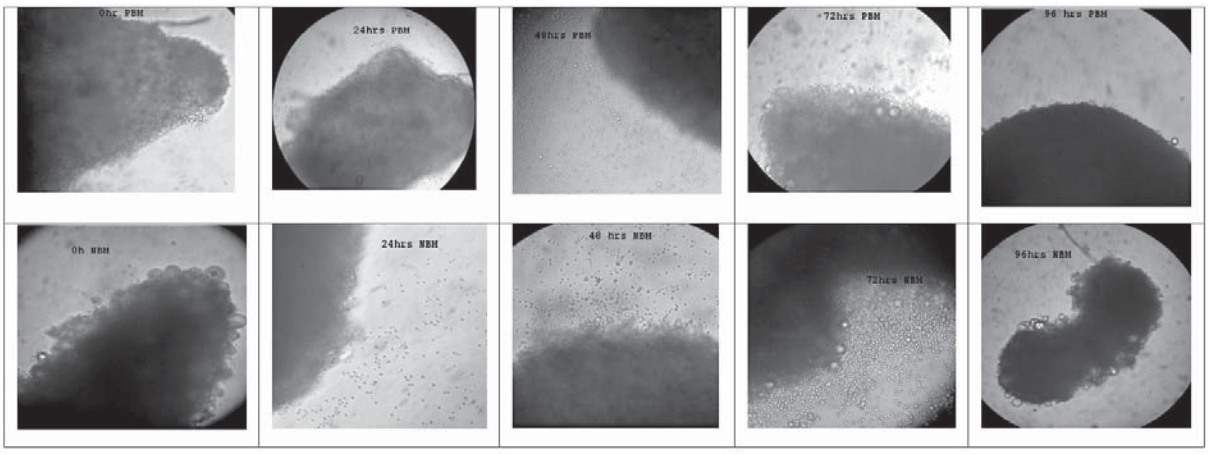

Figure 4 *Short term bone marrow explant culture*
A time-course experimental setup revealed the exact bone marrow microenvironmental status [control (NBM) vs. pesticide treated (PBM)] under the in vitro *short term culture condition where control marrow cell population moved rapidly towards the nutritionally enriched* in vitro *media to thrive and further repopulate in contrast to pesticide-treated marrow.*

Table 2 *Flow cytometrically measured Mean Fluorescence Intensity (MFI) values of Sonic hedgehog (Shh), Patched1 (Ptch1), Smothened (Smo), and Gli1 protein expression in control and experimentally pesticide-treated (aplastic bone marrow) haematopoietic cells.*

Marker	Mean Fluorescence Intensity	
	Treated (N=24)	Control (N=6)
Shh	178.2±2.7	46.35±3.70 #
Ptch1	400±3.2	320.4±1.90 #
Smo	75.85±1.65	40.32±2.01 #
Gli1	550±3.54	365.9±3.45 #

 # Marked values are significantly different (P<0.0001) from the values of the control animals within the same row.

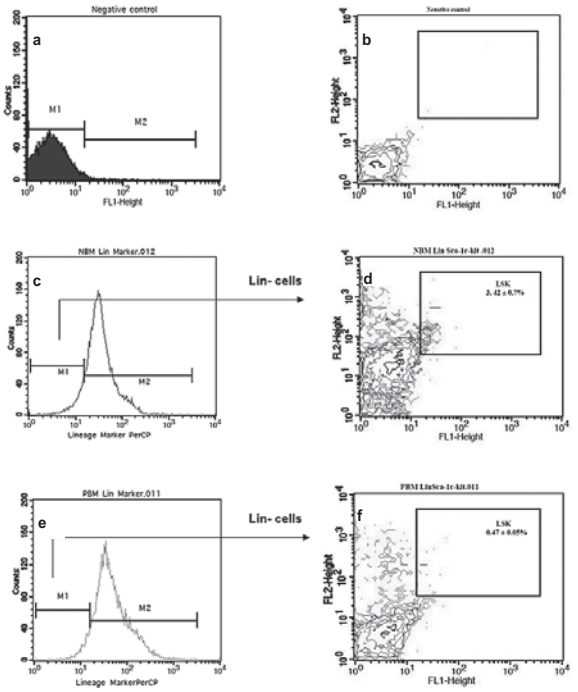

Figure 5 *Flow cytometric analysis of haematopoietic stem and progenitor (LSK) cell population in control and pesticide exposed animals*
Figures 5a to 5f represent a quantitative comparison of primitive haematopoietic stem and progenitor cell population in control (NBM; N=6) and pesticide-treated bone marrow (PBM) from a pooled preparation (N=8 for each experiment repeated three times).

(P<0.0001) (Table 2). Here, MFI of epitope-bound fluorophore-tagged antibodies was directly proportional to the concerned protein expression in the subjected cells measured by flow cytometry (Figures 6a-6d, Table 2).

DISCUSSION

Agrochemicals like pesticides are designed to target agricultural pests but incidentally these

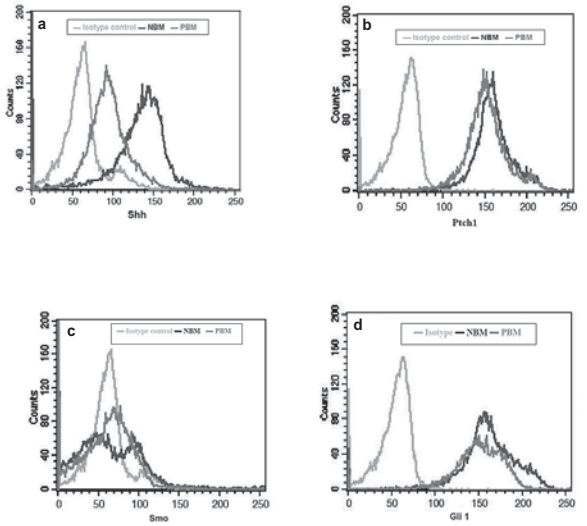

Figure 6 *Flow cytometric evaluations of Hedgehog signalling and its components*
Comparative flow cytometric expression study of Shh and Ptch-1 show a significant amount of downregulation in pesticide-treated bone marrow (PBM) haematopoietic cellular compartment in comparison to control bone marrow (NBM) haematopoietic compartment (Figures 6a and 6b). Smo, another transmembrane component and a positive regulator of Hedgehog signalling pathway also faced downregulation in aplastic condition in comparison with the control group (Figure 6c). Figure 6d shows further downregulation of Gli-1 expression in the aplastic bone marrow. Here, the flow cytometric expression estimation of Hh signalling components was performed on the basis of an analysis gate, which included mostly stem and progenitor population of scattergram. After gating MFI was determined by histogram analysis.

chemicals also harm a broad range of non-targeted organisms including humans. The widespread use of pesticides means we face a continuous risk of exposure to these compounds, which can have several adverse effects. In such cases previous research elucidated the mechanistic correlation of pesticide exposure and neurological disorders. Reports concerning the haematopoietic system, on the other hand, are scanty (4-7, 27-29). Here we considered adult definitive haematopoietic system i.e. bone marrow as our organ of interest to study the mechanism of long term pesticide exposure through inhalation and haematopoietic failure.

The present study revealed an overall diminution of peripheral haemogram parameters and it led us to propose an inference that chronic pesticide exposure hindered neutrophilopoiesis and distorted the morphology of nucleus along with leucopoenia. Low haemoglobin level and diminished total RBC were the ultimate results of impaired erythropoiesis and

generation of low number of reticulocytes, an immediate progenitor of mature RBC, which signified the presence of aplasia in pesticide exposed mice.

On the other hand, a declining state of haematopoietic cells was observed in aplastic marrow smear with scattered lymphocytes and numerous large adipocytes which developed from pre-adipocytic fibroblasts, a special feature of aplastic bone marrow (Figures 1b and 1c). Stromal collagenous fibres were also evident in experimental bone marrow. These large adipocytes and stromal fibres actually replaced the empty spaces of bone marrow produced by long-term pesticide treatment. Furthermore, chronic pesticide inhalation also reduced the total bone marrow cellular components in comparison to the control mice.

Time-bound and comparative bone marrow explant cultures of two aforesaid groups of animals represented a very authentic scenario regarding pesticide exposure and bone marrow cell release pattern under *in vitro* condition. Hypocellularity of pesticide-exposed bone marrow was further established by the present experimental effort. Unlike its normal counterpart, pesticide-exposed bone marrow explant released its cell pool very slowly and in low quantity over time. The low cell-releasing pattern was also an evidence of hypocellularity of bone marrow, which indicated the shortage of marrow haematopoietic cellular storage, low self-renewal, and proliferation activity. Bone marrow explant culture study also exhibited a phenomenon that the pesticide-exposed sample, following a 48-hour lagging period, starts releasing the haematopoietic cells which could not thrive under *in vitro* condition as compared to the normal counterpart. This finding reinforces the fact that chronic pesticide exposure hampers the cellular proliferation rhythm, which ultimately delays the cell release behaviour in comparison to normal bone marrow (Figure 3).

The reduced proliferation, as well as depletion of haematopoietic stem and progenitor cells, added an extra dimension to the reason behind marrow hypocellularity, impaired neutrophilopoiesis, erythropoiesis, and ultimately the occurrence of peripheral blood pancytopoenia. Besides, it also explained how progressive loss of primitive $Lin^{-}Sca\text{-}1^{+}C\text{-}kit^{+}$ (LSK) haematopoietic population in endosteal niche or bone marrow microenvironment of pesticide-exposed mice failed to replenish the pancytopoenia in the vascular niche, as a result of which we observed a significant diminution in peripheral blood elements.

The reason behind the shortage of self-renewing, proliferating haematopoietic stem cells in the bone marrow of pesticide-induced aplastic mice is mostly unexplained. We tried to study the event by our flow cytometric analysis (Figure 6) of the expression pattern of important protein components related to the canonical Hedgehog-Gli1 signalling axis. As we were already aware that hedgehog signalling had a profound effect on embryogenesis and adult tissue homeostasis, we considered the expression status of Shh, Ptch-1, Smo, and Gli1, a transcription factor of the zinc finger family, which activates the hedgehog targeted genes in the haematopoietic stem-progenitor rich compartment. In 2009, Hoffamann et al. (40) and Gao et al. (41) separately reported that the Hh pathway was dispensable for the adult HSC maintenance and differentiation process. However, their findings were not consistent with several studies by Trowbridge et al. (44), Dierks et al. (45), Zhao et al. (46), and Bharadwaj et al. (47). The limitations of their (Hoffman et al. and Gao et al.) studies (40, 41) were discussed in ′The Niche′-Nature Stem Cell Blogs (54) and in a spotlight review of ′Leukaemia′ journal by Aifantis et al. (43) and Merchant et al (42). Irrespective of their controversial studies, we observed sharp downregulation in Hh signalling cascade from the upstream regulator Ptch-1 to the downstream executor Gli-1 along with its ligands Shh in our pesticide-induced aplastic murine bone marrow haematopoietic compartment. It is known to us that Ptch-1 is a protooncogene which affects cell cycle as well as lineage commitment of haematopoietic stem cells (34). In our study, diminished expression of Ptch-1 in pesticide-induced aplastic bone marrow reflected low conversion of primitive LSK population to more mature progenitors and blood corpuscles in peripheral circulation.

Diminution of bone marrow LSK population and downregulation of Smo expression in the pesticide-exposed animals presented a parallel observation with the study of Zhao et al. who showed that $Smo^{-/-}$ and cyclopamine (Smo inhibitor) treatment in mice drastically reduced bone marrow LSK population in comparison to the control (46). Downregulation of Smo expression in our study might be the direct possible manifestation of pesticide exposure. We suspect that the Hexaconazole, a systemic fungicide of the ′azole′ family used in our experiment, possibly acted as an inhibitor for Smo downregulation in pesticide-induced bone marrow failure because it has been found that Itraconazole, another clinically well

known systemic fungicide of the azole family, showed Smo antagonism in a recent study on murine tumour (16). Smo failed to transduce the signal up to the final downstream executor Gli1, which was seen as a usual depressive status in pesticide-induced aplastic bone marrow in contrast to its normal counterpart (Figure 6).

Reduced Gli1 expression also affected the hedgehog pathway by downregulating the intracellular feedback loop of Ptch1 and Gli1. Under normal conditions, Gli1 protein helps to transcribe Gli1 by means of a positive feedback mechanism and also upregulates the signalling repressor Ptch1, a negative feedback that reins the Gli1 regulatory loop. It actually represents a signalling network composed of a positive transcriptional feedback loop embedded within a negative signalling feedback loop.

Our present experimental data revealed a steady dampening condition in the Gli1–Ptch1 feedback loop, in which downregulation of Gli-1 gene product automatically suppressed the Ptch-1 expression by direct action of pesticide-deregulated Smo repression. Henceforth, it is clear that chronic pesticide exposure also hampers the direct feedback loop between Gli-1 and Ptch-1 inside the bone marrow haematopoietic stem/progenitor cell population, in the context of Smo downregulation, which leads to a progressive degeneration of haematopoietic machinery. In these contexts, the altered internal regulatory loop between Ptch1-Smo-Gli1 of Hedgehog signalling crippled the self-renewal, proliferation, and differentiation capacity of haematopoietic stem cells and resulted in bone marrow suppression. However, future studies might be designed to further elucidate the role of Hedgehog signalling in the pathology of the pesticide-induced bone marrow toxicity by constitutive over-expression of the active components of the Hh signalling pathway in the stem cells. Only then could one determine whether this approach rescues the marrow failure observed in our established animal model.

CONCLUSION

At the end we conclude that the unresolved paradox of the development of intrinsic stem cell defect of acquired aplastic anaemia through chronic pesticide exposure lies in the deregulation of the canonical Hedgehog-Gli1 signalling cascade. Besides, commercially available pesticides are harmful not only to the nervous system of the non-target organisms but also to other developmentally important signalling cascades like Hedgehog signalling, which functions as “stem cell fate switch”. Finally, our experiment suggests the possibility of using primarily Gli-1 as the biomarker for those patients who are chronically exposed to pesticides in agricultural field and are either suffering from aplastic anaemia or having propensity towards the development of bone marrow aplasia.

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Sažetak

PROMIJENJENI KANONIJSKI SIGNALNI PUT HEDGEHOG-GLI KOD PESTICIDIMA INDUCIRANE APLAZIJE KOŠTANE SRŽI ISPITAN NA MIŠJEM MODELU

Mehaničko međudjelovanje izlaganja pesticidima i razvoja aplazije koštane srži još uvijek nije u potpunosti utvrđeno, ali postoje naznake da kronično izlaganje pesticidima u nekim slučajevima može uzrokovati aplaziju koštane srži poput hematopoetskoga degenerativnog stanja u ljudi. Kanonijski signalni put Hedgehog (Hh) ima višestruke uloge u mnogim razvojnim procesima, uključujući i hematopoezu. Ovo je ispitivanje imalo za cilj istražiti status četiri glavne sastavnice kanonijskoga signalnoga puta Hedgehog, Sonic Hedgehog (Shh), Ptch1, Smo i Gli1, na mišjem modelu pesticidima inducirane aplazije koštane srži. Koristili smo 5 % vodenu mješavinu pesticida (klorpirifos, profenofos, cipermetrin, alfa-metrin i heksakonazol) kojoj smo miševe izložili udisanjem i preko kože tijekom 6 sati dnevno i 5 dana tjedno do najviše 90 dana. Kronično izlaganje pesticidima vezano uz aplaziju koštane srži bilo je primarno potvrđeno krvnom slikom, celularnošću koštane srži, kratkotrajnom kulturom eksplantata koštane srži radi stanične kinetike, razmazom koštane srži i ekspresijskim obrascem protočne citometrije izvanstaničnog receptora Lin-Sca-1^{+}C-kit^{+}. Potom su analizirane sastavnice signalnog puta hedgehog u koštanoj srži kontrolnih jedinki i aplastičnih životinja koje su tretirane pesticidima. Rezultati su pokazali pancitopeniju periferne krvi, razvoju anomaliju neutrofila, depresiju primitivnih matičnih stanica i prastanica uz Shh, Ptch1, Smo i Gli1 ekspresiju u skupini koja je imala aplaziju. Ovo istraživanje navodi na zaključak da pesticidi uzrokuju sniženje dvaju kritičnih proteina - Ptch1 i Gli1 - unutar hematopoetskih matičnih stanica i prastanica uzrokujući time hematopoetsku homeostazu i poremećaje regeneracijskog mehanizma *in vivo* zajedno s aplazijom koštane srži.

KLJUČNE RIJEČI: *hematopoetske matične stanice, "patched" protein, "smoothened" protein, "sonic hedgehog" protein, supresija koštane srži*

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