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Inflammation: A protagonist in development of carcinogen induced cervical cancer in mice

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Inflammation– induced systemic stress plays an essential role in neoplastic progression. Chronic exposure to chemical carcinogens can induce persistent inflammatory changes which further augment loss in physiological hormesis of an organism thereby favouring carcinogenesis. The present study investigated the role of inflammation and associated systemic stress in the development of cervical carcinoma in a 3-methylcholanthrene (3-MC; a chemical carcinogen) induced *in vivo* cervical cancer model. When the cervix of 5-6 weeks old virgin female Swiss Albino mice (*Mus musculus*) was treated with 3-MC (0.6 mg/mL), remarkable alteration in its cervical cytopathology was observed. An increase in duration of 3-MC treatment caused an outburst in the number and variety of infiltrating granulocytes and agranulocytes in mice cervix. Thus, a high leukocyte index was indicative of prevalent cervical inflammatory changes. Elevated activities of SGPT, SGOT, serum alkaline phosphatase enzymes along with the presence of elevated serum creatinine levels suggested liver and renal dysfunctions. These observations were supported by alterations in hepatic histopathology of 3-MC treated mice. Surged activities and expression profiles of inflammatory cytokines (IL-6 and IL-8) in cervix tissue had conclusively established the crucial role played by inflammation– mediated systemic stress in favouring the development of cervical cancer in a carcinogen-induced *in vivo* model.

Keywords: Cervical cancer, Chronic inflammation, Cytokines, Dysplasia, *Helicobacter pylori*, Methylcholanthrene, Systemic stress

The concept of an intricate association between inflammation and cancer dates back to 19th century as chronic inflammation triggers mechanisms underlying the progression of cancer[1]. Proposed to be one of the key factors responsible for initiating the malignant transformation, inflammation is also termed as the seventh hallmark of cancer[2]. Chronic inflammation plays a crucial role in enriching the tumour microenvironment with prosurvival signals which enables the neoplastic cells to survive and proliferate by evading the adaptive immune response of the host and by developing chemoresistance[3,4]. Sometimes localized inflammation mediated by a persistent bacterial and viral infection can lead to increased cancer risk. Unremitted *Helicobacter pylori* infections of the stomach may result in gastric adenocarcinoma along with lymphoma of the MALT (mucosa-associated lymphoid tissue)[5,6]. Although, inflammation can act as a "two-edged sword" because, in some diseases like psoriasis, the presence of a steady–state of inflammation helps in reducing the disease[7,8]. Conversely, carcinogens like polycyclic aromatic hydrocarbons (PAHs) or various aromatic amines render their carcinogenicity by triggering chronic inflammation which furthers tumorigenesis[8,9]. The whole episode of cancer– related inflammation is dictated over by the transcription factor NF-κB and its relative signal transducers such as COX2, IL-6, and IL-8[10-12]. These inducers of malignant transformation drive the process of neoplasia by bringing about subsequent deregulation of tumour suppressor genes (p53, p21, and Rb) followed by upregulation of proliferative markers (Ki-67 and PCNA)[13,14].

3-methylcholanthrene (3-MC), a carcinogen belonging to the family of PAHs is known to cause

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Abbreviations: 3-MC, 3- methylcholanthrene; HH, Harris Haematoxylin; IL-6, interleukin-6; IL-8, interleukin-8; LI, leukocyte index; MALT, Mucosa-associated lymphoid tissue; NBF, Neutral Buffered Formalin; PAH, polycyclic aromatic hydrocarbon; PCNA, Proliferating cell nuclear antigen; RNS, Reactive nitrogen species; ROS, Reactive oxygen species

the cancer of many organs such as skin, lungs, and cervix[15]. Cervical cancer, the 4th most leading cause of mortality and morbidity amongst women worldwide is predominantly caused by high– risk human papilloma viruses[16-18]. Epidemiological reports suggest that PAHs can also increase cervical cancer risks in women[19]. 3-MC like other PAHs contributes to carcinogenicity while it gets metabolically activated by phase I and phase II enzymes such as cytochrome P-450(CYP450), catechol-O-methyltransferase, epoxide hydrolase, peroxidases, glutathione S-transferases, N-acetyltransferases and sulfotransferases[20]. 3-MC metabolites such as epoxy derivatives mediate free radical bursts[21]. It induces excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) production[22,23]. These free radicals are electrophilic species which attack the intracellular nucleophilic moieties such as the DNA and glutathione and thereby promote cytotoxicity by induction of deleterious mutations that impair the repair machinery of the cells[24-26]. In this regard, 3-MC have been reported to be used for development of *in vivo* cancer models[27,28].

It is already known that cervical carcinoma induced by 3-MC is characterized by constitutive expressions of stress– related biomarkers[29]. Based on these previous reports; the present study has been designed to investigate the role of systemic stress induced by persistent inflammatory mediators produced by chronic 3-MC treatment in Swiss Albino mice, while the development of an *in vivo* cervical cancer model.

Materials and Methods

Animal maintenance

5-6 weeks old virgin female Swiss Albino mice (*Mus musculus*) weighing about 23-25 gms were obtained from Central Animal Facility of Chittaranjan National Cancer Institute (CNCI). These mice were kept in polyvinyl cages which were maintained in a well-ventilated room (temperature-22±2°C; relative humidity: 50-60%) following 12 h day and night cycle. The acclimatization of animals (mice) was carried out for 2 weeks prior to treatment. All –female mice were kept in complete isolation from male mice. This aided in nullifying hormonal interference by bringing about pheromone influenced synchrony of their oestrous cycles[30]. All the animal experimentation was carried out in accordance with the animal ethics guidelines of the Institutional Animal Ethics Committee (IAEC,CNCI) certified by the Committee Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. Food and water were given *ad libitum* which means free feeding.

Experimental design

Mice were randomized into 3 major groups; where 6 mice were kept in each major group. Group I was kept as a control group which received no treatment, Group II was maintained as a vehicle control batch where mice were treated with petroleum ether (PE) solely, while Group III was designated to study the progression of cervical carcinogenesis in mice which underwent chronic treatment with a carcinogen namely 3-MC (MP Biomedicals/Fisher Scientific, USA) dissolved in petroleum ether for a time span of 30 weeks, respectively. The concentration of the 3-MC solution used for the study was 0.6 mg/mL. This dose of a carcinogen was selected as a safe dosage for treatment after dosimetry. Food and water were provided *ad libitum* to all three groups.

The procedure followed for carcinogen treatment is a modification of Murphy's String method[31]. The carcinogen solution (3-MC dissolved in petroleum ether) was flushed over the cervix of the animals belonging to group III and petroleum ether (vehicle solvent) to group II, respectively. During the continual treatment process, animals were monitored for possible visible health abnormalities and death. Animals from each of these groups were sacrificed intermittently (6, 12, 18, 24 and 30th week) by cervical dislocation followed by the collection of organs and blood for experimental study. All the experiments were repeated thrice.

Record of body weight

Weekly body weight records of animals were maintained to ascertain the impact of the chronic carcinogen treatment on their physiology.

Cytopathological study

Phosphate Buffered Saline (PBS, pH-7.4) was dropped on a clean grease- free glass slide on which cervical exfoliated cells were smeared into a thin conventional layer after collection. Cervical smears were stained as per the polychromatic staining method of the Papanicolaou staining (Pap staining) process[32]. The cell smears were fixed in 100% ethyl alcohol followed by subsequent staining with Harris

Haematoxylin (HH), Orange G6 (OG6) and Eosin Azure (EA50) [Merck Millipore, Mumbai, India]. Excess haematoxylin stain was removed by rinsing the slides in tap water whereas an excess of OG6 and EA50 was washed in 70% and 90% alcohol, respectively followed by clearance in 100% ethyl alcohol and xylene. Thereafter the slides were mounted and observed under a light microscope (Zeiss).

Enumeration of leukocyte index

A leukocyte index was prepared by calculating the ratio of the number of leukocytes observed to the total number of cervical exfoliated cells present in a particular field of the Pap smear for each treatment. More than 20 fields were examined for each slide and data were recorded to enumerate leukocyte index (LI), LI = [(number of leukocytes observed/total number of cervical exfoliated cells)]. The study of the differential subpopulations of leukocytes infiltrating the cervical region during the treatment course was also performed.

Estimation of liver enzymes

The hepatic toxicity induced because of chronic carcinogen treatment was assessed by estimating serum SGOT, SGPT, and alkaline phosphatase by using AUTOSPAN liquid stable kits[33,34]. After the sacrifice of mice, blood was withdrawn from the heart's ventricular apex using a syringe and collected in serum separating vials. The serum was separated from blood using these vials.

Estimation of serum creatinine

Serum creatinine levels were measured by Jaffe's method[35]. The results were expressed in terms of fold increase in creatinine levels so that a comparative study can be made. All the experiments were repeated thrice.

Quantification of IL-6 and IL-8 in cervix tissue lysate

Cytokines, IL-6 and IL-8 were determined by using the cervical tissue lysates by using commercially available ELISA kits (Invitrogen). The optical density of the coloured solution was measured using the ELISA reader (TECAN) at 450 nm. The results were recorded against a standard blank. The experiment was repeated thrice.

Western Blot analysis

Lysates from cervix tissue were used to measure the expression of IL-6 and IL-8 by Western blotting using specific antibodies. The concentration of proteins of cervical tissue lysates of both normal and tumour bearing mice was estimated by Lowry's method. Electrophoresis through 12.5% SDS-polyacrylamide gels was performed where 50 µg of total protein was loaded into each well using an electrophoresis buffer (25 mM Tris, 192 mM glycine, 10% SDS) within the electrophoretic chamber. The separated proteins were electrotransferred to nitrocellulose membranes using transfer buffer (250 mM Tris, 192 mM glycine, 10% Methanol). After proper blocking with bovine serum albumin (BSA), membranes were washed properly with Tris Buffered Saline (TBS) pH-7.5 containing 25 mM Tris-HCl and 150 mM NaCl followed by incubation with primary antibodies overnight at 4°C with constant shaking. The blots were washed 4 times with TBST Buffer (40 mL TBS and 20 µL Tween-20) solution following incubation with secondary antibodies conjugated with alkaline phosphatase enzyme (1:1000 dilutions in TBST) at 4°C for 2 h. Membranes were then washed 4 times and incubated with the substrate (BCIP/NBT) to visualize the proteins in dark. These experiments were repeated thrice.

Histopathological study

Liver tissue was dissected out and washed in cold normal saline (0.87%). This was fixed in 10% Neutral Buffered Formalin (NBF) for 24 h after which it was processed through grades of alcohol and xylene followed by paraffin embedding for 1hr. Later, this embedded tissue was taken for paraffin block preparation followed by microtomy. Tissue sections of 5 µM thickness were made using microtome and stretched over a glass slide which was taken for haematoxylin and eosin staining after removal of paraffin in xylene followed by treatment in ascending and descending grades of alcohol[36]. After staining the sections, these slides were immersed in xylene following which they were mounted and examined under a light microscope (Zeiss).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software. A Comparison between the three groups was done by ANOVA, followed by the student's *t*-test. A *P*-value <0.005 was considered statistically significant.

Results

Effect of carcinogen treatment on the body weight of the animals

Chronic treatment with 3-MC resulted in gradual

loss in bodyweight of mice. This observation was noted for up to 12 weeks of treatment. As the treatment duration reached 16 weeks, a conspicuous rise in the body weight was observed which was maintained till 30 weeks of carcinogen treatment (Fig. 1). No such fluctuation was observed in the bodyweight of the mice of control and vehicle control batches.

Effect of 3-MC on infiltrating inflammatory cells in the cervix

Pap smear test with cervical swabs revealed that treatment of mice with 3-MC in a chronic fashion for 30 weeks caused infiltration of various multitudes of inflammatory mediators such as granulocytes and agranulocytes in their cervical region. During the initial phase of 3-MC treatment, eosinophils were sparsely present with a LI ratio of 4±0.2 and thus exhibited no significant alteration for batch (Fig. 2A & D). As treatment duration reached 12 weeks, neutrophils appeared in excessive numbers with a significantly high LI ratio of 8.5±0.176 in comparison to control mice (Fig. 2B & D). Monocytes joined the milieu of infiltrating cervical leukocytes when the carcinogen treatment reached 24 and 30 weeks, respectively (Fig. 2C). The LI ratio during the 24th week of 3-MC treatment was found to be as high as 10.2±0.34 and it remained almost unchanged with a little significant rise (11.8±0.18) during the 30th week. Although when compared to control and vehicle control groups, these LI values for both 24th week and 30th week were found to be extremely high. However, the LI value of the vehicle control group exhibited no significant difference with that of the control batch indicating the absence of any contribution of PE in inducing inflammation in the mice cervix (Fig. 2D). Furthermore, increased treatment duration conciliated an increase in the total count of leukocyte infiltrating the cervix (Fig. 2D). This indicated that 3-MC induced persistent inflammatory changes in the cervix might have paved the way for cervical carcinogenesis.

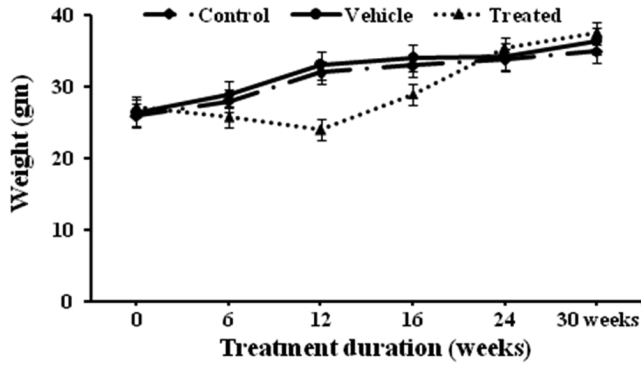

Fig. 1 — Trends in body weight fluctuations as represented graphically. Mice treated with 3-MC underwent gradual loss in body weight till 12th week followed by a gain in body weight. The appearance of tumour near the cervix was observed in most of the treated mice after on an average of 16th week of treatment

Activity and expression of inflammatory mediators (IL-6 and IL-8) in the cervix tissue during carcinogenic progression

Owing to the increased infiltration of leukocytes observed in the cervix of 3-MC treated mice, the activity of IL-6 and IL-8 was studied. The subsequent rise in IL-6 and IL-8 activity was found in the cervix with an increase in treatment duration (Fig. 3A). These cytokines are essential for the maturation and differentiation of neutrophils. Activities of both the cytokines (IL-6 and IL-8) were found to rise 6th week onwards of 3-MC treatment in comparison to untreated control (Fig. 3A). A surge in IL-6 activity in the cervix of animals with a fold increase value of 81.2±0.73 was noted during the 24th week of treatment while an escalation of 79.7 ±0.40 in IL-8 fold increase activity was found during 18th week of treatment (Fig. 3A). No significant alteration in IL-6 and IL-8 activity was however observed (IL-6 fold: 3.9±0.193; IL-8 fold: 5.2 ±0.09) in control animals. IL-6 and IL-8 activities were extremely high in the mice cervix which had received 30 weeks of 3-MC

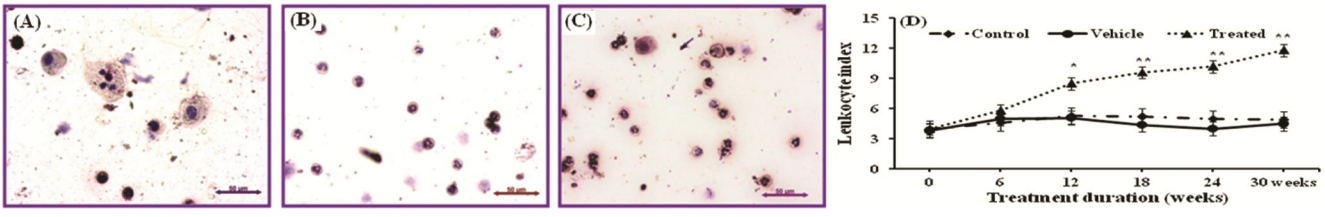

Fig. 2 — Differential subpopulations of leucocytes infiltrating the cervical region during chronic 3-MC treatment as observed in Pap smear. (A) appearance of eosinophils in the cervix during initial phase of treatment; (B) increased infiltration of neutrophils during 12 weeks of 3-MC treatment; (C) emergence of monocytes alongside neutrophils during 24-30 weeks of 3-MC treatment; and (D) Graphical representation of leucocyte index (LI) as calculated as the ratio of number of leucocytes observed to the total number of exfoliated cells present in each field of Pap Smear. Values are mean ± SD, (n=6). *P** <0.005 and *P*** <0.0001 *vs* untreated control batch

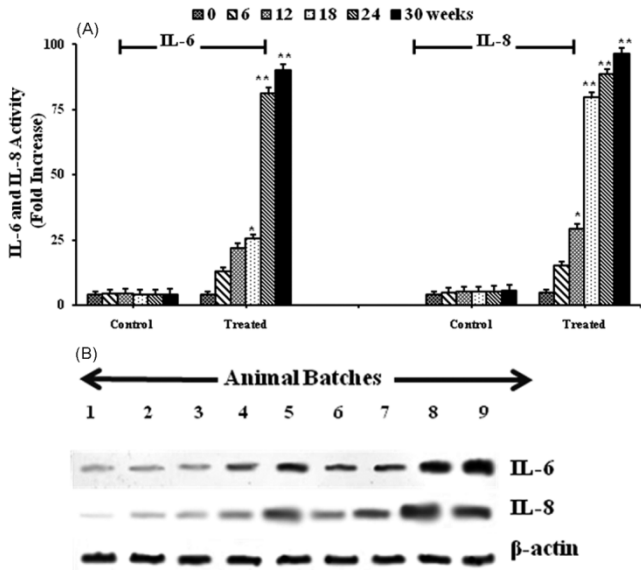

Fig. 3 — Activity and expression profiles of IL-6 and IL-8 in control mice and mice group subjected to chronic 3-MC treatment at the cervix. (A) IL-6 and IL-8 levels from cervical tissue lysates. Values represent mean ±SD (n=3). *P** <0.005 and *P*** <0.0001 *vs* controls; and (B) Protein expression of IL-6 and IL-8 as observed by western blotting. Lane 1: control batch, lane 2: vehicle control, lane 3: 3-MC treatment for 6 weeks, lane 4: 3-MC treatment for 12 weeks, lane 5: 3-MC treatment for 18 weeks, lane 6: 3-MC treatment for 24 weeks, lane 7: 3-MC treatment for 30 weeks (tumor- bearing cervix), lane 8: corticomedullary region of the tumor obtained after 30 weeks, lane 9: richly vasculated medullary region of the tumor. β-actin was used as a control to ensure equal protein loading

treatment (Fig. 3A).

Western Blot analysis to study the expressions of IL-6 and IL-8 from the isolated cervix tissues of both normal and tumour bearing mice were performed to ascertain the putative role played by these cytokines in carcinogenic progression (Fig. 3B). In control and vehicle control group expression profiles of IL-6 and IL-8 were very poor, which increased concomitantly with increased treatment duration in the treated group (Fig. 3B). Expression profiles of both IL-6 and IL-8 strongly corroborated with the activities of these cytokines.

Liver dysfunction during carcinogenic progression

The next objective of the study is to ascertain whether chronic exposure to 3-MC led to distorted liver function in treated mice. Biochemical assays like an estimation of SGPT and SGOT were carried out using kits. The results were expressed as IU/L which is the product of a change in absorbance of the sample per minute and kinetic factor (provided along with the kit protocol). An uprising trend of SGPT and SGOT enzyme activities were observed after 4 to 5 weeks of 3-MC treatment, which were found to surge significantly (SGPT: 12.3 IU/L±0.261; SGOT: 20.62 IU/L±0.759) by 6^{th} week of 3-MC treatment onwards to control (Fig. 4A). This escalation of enzymatic activities exhilarated as the treatment process progressed through 12^{th}-16^{th} week, eventually reaching to stasis by 24^{th} to 30^{th} weeks (Fig. 4A). On the contrary, no significant change was observed between the SGPT and SGOT kinetics of vehicle control and control animals (Fig. 4A).

High SGPT and SGOT activities were found to be coherently associated with the alterations in hepatic histopathology of 3-MC treated animals (Fig. 4B & C). The presence of inflammatory infiltrates alongside clusters of necrotic hepatocytes and damaged portal tract with bile duct proliferation and marked atypia was well noted in the liver histological sections obtained from animals that had received 30 weeks of 3-MC treatment (Fig. 4B). Normal central veins were observed in liver sections from the untreated control group with no evidence of hepatocyte injury, fibrosis or dysplasia and malignancy (Fig. 4C).

Serum Alkaline phosphatase enzyme activity

A gradual increase in serum alkaline phosphatase (ALP) enzyme activity with an increase in the duration of 3-MC treatment was observed. There was no change in the serum enzyme activity documented during the initial 6 weeks of 3-MC treatment to control mice (Fig. 5A). During the 12^{th} week, the serum alkaline phosphatase kinetics for treated mice was recorded as 3.4 IU/L±1.032. This was extremely higher when compared to control mice. A significant increase in enzyme activity was apparent when treatment duration was increased from 12 weeks to 30 weeks (Fig. 5A). Alkaline phosphatase enzyme activity for vehicle control and control batches conversely exhibited no significant alteration (Fig. 5A).

Estimation of serum creatinine levels

Chronic 3-MC treatment till 30 weeks led to impairment of renal function as comprehended from the presence of high levels of serum creatinine. Consistent elevation in the level of serum creatinine was found to be maintained in treated groups between 12 to 30 weeks of treatment (Fig. 5B). During the initial phase of treatment of up to 6 weeks, no significant change in serum creatinine level was observed in comparison to control mice. Changes were found to be gradual with a subtle significant fold increase in serum creatinine levels by 2.4 ±0.072

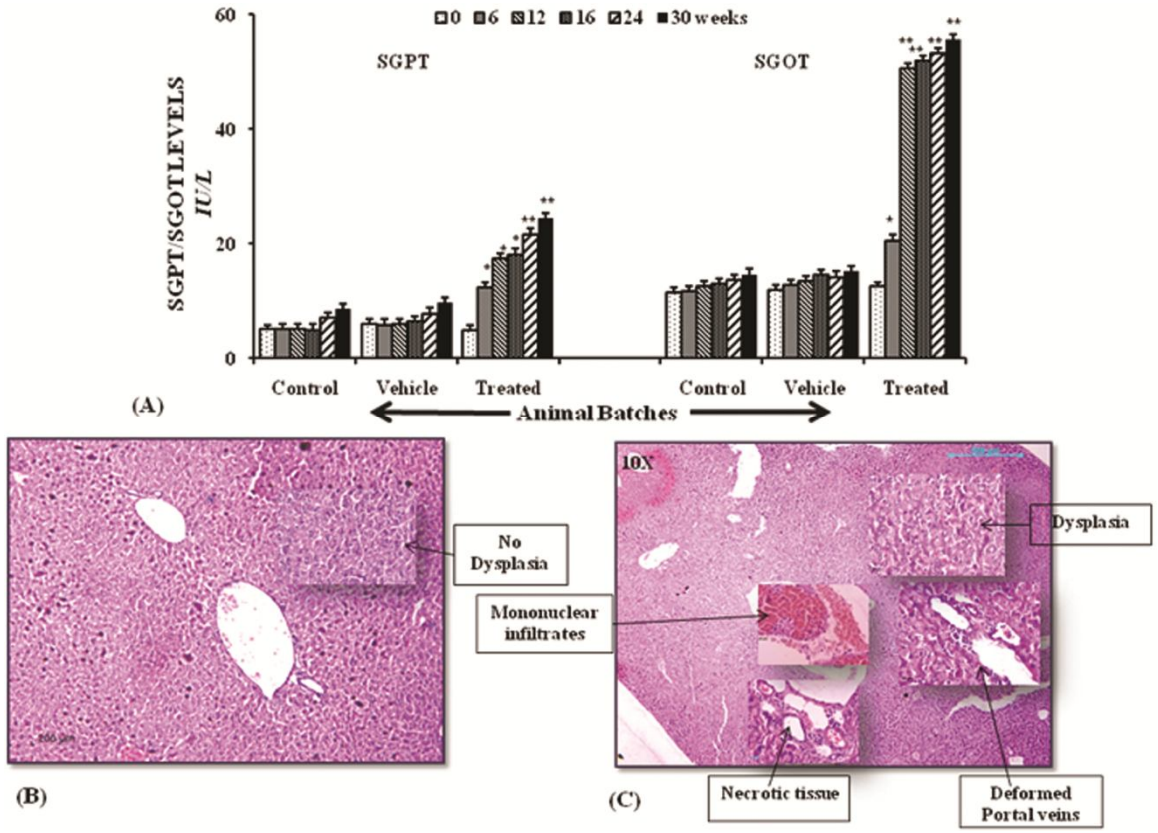

Fig. 4 — (A) Serum SGPT and SGOT levels in control, vehicle and 3-MC treated groups. **P* <0.005 and ***P* <0.0001 *vs* controls; (B) Histological (H & E staining) observation of liver from the untreated control group, showing normal hepatic architecture; and (C) Abnormal histopathology showing deformed central vein, damaged portal tract, remarkable atypia, and dysplasia after treatment with 3-MC for 30 weeks. Magnification: 10X

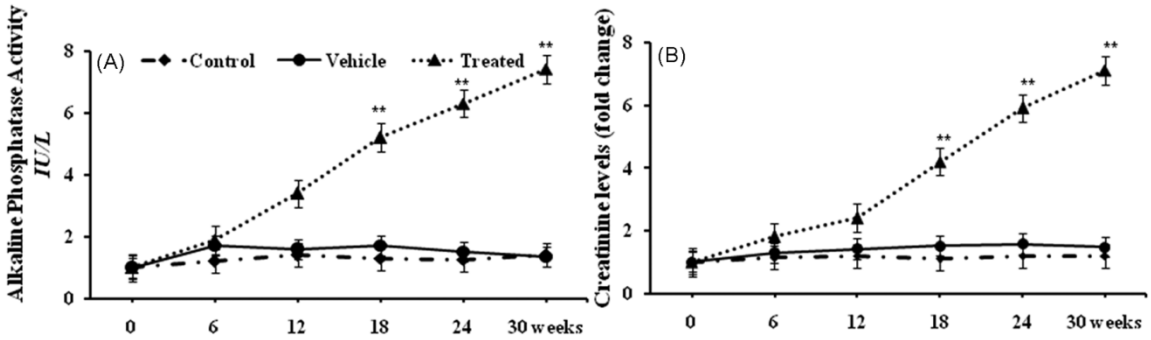

Fig. 5 — (A) Serum alkaline phosphatase kinetics; and (B) serum creatinine level estimation. Values (mean ± SD) for both the parameters measured in the serum of mice treated chronically with 3-MC at 0, 6, 12, 18, 24 and 30 weeks. ***P* <0.001 *vs* control

being documented in the 12th week of 3-MC treatment (Fig. 5B). Creatinine levels in the serum obtained from the mice of vehicle control and control batches did not exhibit any difference (Fig. 5B).

Discussion

Metabolic transformation is a prerequisite for 3-MC to mediate the process of carcinogenesis in an organism. Like other PAHs, 3-MC also gets metabolized by the liver CYP450 leading to the generation of electrophilic moieties which induces DNA damage. Administration of 3-MC in *in vivo* model of cervical cancer was although limited only to the cervical region, its effect was not only conspicuous in the cervix but also in other organs. Evidence to this fact has been recorded in terms of

gradual loss in body weight of the treated mice particularly till the 12th week of 3-MC treatment. Since the treatment was made on a chronic basis, the accumulation of 3-MC metabolites might have intervened into the normal metabolic trends of these mice. This had driven the process of tumorigenesis in the cervix by the 24th week of 3-MC treatment. As the treatment progressed to 16 weeks, there was a gain in body weight observed which continued till the 30th week of treatment.

The high rate of infiltration of leukocytes in the cervical region indicated the initiation and prevalence of inflammation. The presence of neutrophils in large numbers had played an important role in the development of cervical cancer in mice due to chronic 3-MC treatment. High numbers of neutrophil in blood in comparison to other leukocytes are considered to be a vital factor for cancer prognosis[37]. Neutrophils are the major drivers of localized inflammation which releases chemoattractants for other inflammatory cells[38]. In this study appearance of monocytes during the later phases of 3-MC treatment is also suggestive of the role played by neutrophil in the maintenance of induced inflammation. This remodelled the cervical niche to allow neoplastic growth.

During the developmental stages of *in vivo model*, inflammation- mediated carcinogenesis was found to be associated with higher expression profiles of IL-6 and IL-8 as obvious from western blot results. Activities of these cytokines in the cervix tissue lysates were also found to be quite high. In HPV-16 mediated cervical carcinogenesis, IL-6 titres are found to be comparatively higher in invasive cervical carcinoma stages than in precursor lesions[39,40]. IL-6 strengthens the scenario of inflammation by fostering monocyte chemoattractant protein-1 which helps in recruiting monocytes to the site of malignancy[41]. This signalling axis further activates IL-8 which is also known as neutrophil-activating factor. A community signalling loop including IL-6, IL-8, and other inflammatory mediators get formed thereby making the scenario of inflammation more fierce[42]. In HPV mediated cervical carcinogenesis, compelling evidence suggest the mediating the generation of oxidative stress helps to mediating the overactivity of these cytokines that induce cervical carcinogenesis[43,44]. Similar prosurvival signalling might have got manifested in the present study during the development of the *in vivo* model of carcinoma which might be due to a long period of subjection to stress owing to which higher frequency of granulocyte and agranulocyte infiltration was observed in the cervical region. Various reports suggested that excerbating levels of inflammatory cytokines are necessary for progression of cervical cancer as well as for other cancers like colorectal cancers[45-47].

Metabolism of 3-MC always takes place in the liver microsomes[48]. Thus, excess of carcinogen-induced stress can lead to distorted liver function. This serves as an important factor that contributes to the development of conditions favouring the establishment of carcinoma. Major determinants of liver function are SGPT, SGOT and ALP[49-51]. High serum SGPT, SGOT and ALP levels as observed in the present findings indicate despaired liver function which helps in assessing the impact of the stress- induced by carcinogen on the physiology of the treated mice. In case of development of an *in vivo* carcinoma model, determination of SGPT, SGOT, and ALP levels in the serum not only aids in understanding the extent of induced systemic stress but also provides a tool to select the sublethal dosage of carcinogen for treatment[52,53]. Several other researchers have authenticated that administration of carcinogen to mice in a chronic fashion, can induce necessary hepatic toxicity which can serve as a prerequisite to continue the process of carcinogenesis[54,55]. Biochemical changes were found to be coherently associated with the histological architecture of the liver obtained from the same groups of mice. On the other hand, the presence of renal dysfunction is confirmed by high serum creatinine levels.

Conclusion

All these results cumulatively indicated that chronic carcinogen treatment resulted in the induction of a prolonged inflammatory state in the cervix. This acted as an additive stressor for augmenting the loss of physiological homeostasis in the treated mice. Localized treatment of the cervix with 3-MC ultimately culminated in impairment of functions of vital organs like liver and kidney. Thus carcinogenic progression in the cervical carcinoma model *in vivo* is the resultant of the interplay of different inflammatory stressors within the system.

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Conflict of Interest

All authors declare no conflict of interest.

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