

## EFFECT OF IRON OVERLOAD ON 7, 12-DIMETHYLBENZ (A) ANTHRACENE-INDUCED SKIN TUMORIGENESIS

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### ABSTRACT

Iron overload is known to occur in the West European and American population due to the consumption of iron-rich diets. On the other hand, genetic disorders leading to iron overload are also known. Iron overload leads to increased peroxidation and disruptive disintegration of lipid-rich membranes, and predisposes humans for an enhanced risk of cancer induction. In experimental animals iron overload enhances intestinal, colonic, hepatic, pulmonary and mammary carcinogenesis. In this study, we have shown that iron overload is a mild tumor promoter in mouse skin. Female albino Swiss mice were iron overloaded and their backs were shaved. Tumors were initiated using a complete tumorigenesis protocol by applying 200 µg 7,12-dimethylbenz(a) anthracene (DMBA)/mouse in multiple doses of 40 µg DMBA/day for 5 consecutive days. The appearance of the first tumor (latency period), percent of tumor incidence and number of tumors/mouse were recorded. When compared to the positive control group, the iron overloaded mice showed an increased incidence of tumors. In iron overloaded animals, the tumors appeared about four weeks earlier. The number of tumors per mouse were significantly higher in the iron overloaded group. Biochemical studies performed in the present study include the determination of the activity of lipid peroxidation, catalase and xanthine oxidase measurement in mice skin tissue. We observed an iron-mediated induction in lipid peroxidation (LPO) and xanthine oxidase (XOD) and diminished catalase (CAT) activity in skin tissues of mice overloaded with iron as compared to the normal unloaded control group. Based on these studies we propose that iron increases tumor promotion potentials significantly. An induction in LPO in the iron overloaded group suggests that oxidative stress may be responsible for such an observed augmentation of cutaneous carcinogenesis in mice. Our data indicate that iron overload exerts tumor promoting potential in mouse skin, and that oxidative stress generated by iron overload is responsible for the augmentation of cutaneous tumorigenesis.

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## INTRODUCTION

In developing countries, iron deficiency is an important nutritional problem. In developed meat eating countries, however, iron excess may be more of a problem than iron deficiency.<sup>1,2</sup> Iron overload has been observed in the human population throughout the world. It has been estimated that 0.2 to 0.5% of humans have a genetically determined form of iron loading called hereditary hemochromatosis.<sup>3-5</sup> In addition, iron overload has also been observed in patients with thalassemia major.<sup>6,7</sup> In sub-saharan African countries, clinically and pathologically significant iron overload can result from the ingestion of home brewed beer which is rich in iron.<sup>8,9</sup> Porphyria cutanea tarda, chronic liver diseases and chronic renal failure result in patients with significant degrees of iron overload.<sup>7,8,10</sup> Epidemiological studies suggest that increased body iron stores may be associated with an increased risk of cancer and increased overall death rates.<sup>11,12</sup> In experimental animals increased body iron has been shown to enhance mammary,<sup>13</sup> colonic,<sup>14</sup> intestinal<sup>15</sup> and hepatic<sup>16</sup> carcinogenesis. Exposure to iron-containing ores increases the risk of lung cancer<sup>17</sup> and an increased incidence of lung cancer has been observed among smokers having high iron overload.<sup>18,19</sup> These studies suggest that iron overload conditions may prime tissues for an enhanced incidence of carcinogenesis. However, to the best of our knowledge, no evidence has been provided to show that iron overload augments skin carcinogenesis. In this study, we provide evidence that iron overload acts as a cutaneous tumor promoter and enhances DMBA-induced skin carcinogenesis.

## MATERIALS AND METHODS

### Chemicals

Iron dextran (imferon) was purchased from Rallis India, Ltd. Hair removing cream was from Anne French, Geoffrey Manners and Co. Ltd. (India). 7,12-dimethylbenz(a)anthracene (DMBA), and metal iron in powder or crystal form was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Three standard reference materials have been obtained from the International Atomic Energy Agency (Vienna, Austria) to be used as reference for iron estimations. These include 1) Mixed Human Diet, H-9, 2) Rye Flour, V-8, and 3) Hay powder, V-10. Nitric acid, perchloric acid and sulfuric acid of "analar" grade were procured from S.D. Fine Chemicals, E. Merck, and Glaxo (India), respectively. All other chemicals and biochemicals used in this study were either of analytical grade or of highest purity grade available commercially.

### Animals

Swiss albino mice free from pathogen were procured

from the Central Animal House Facility of Hamdard University and used throughout this study for our experiments. Animals were housed in an air conditioned room in polypropylene cages. They had free access to pellet diet (Lipton, India, Ltd.) and water *ad libitum*. For long term chronic experiments, usually 20 mice were housed and for acute experiments, six mice were taken in each group. The animals were kept at a room temperature of 24°C ( $\pm 2^\circ\text{C}$ ) and were exposed to alternate cycles of 12 hour periods of light and darkness. The dorsal skin of the mouse was shaved with electric clippers (Oster A<sub>2</sub>) followed by the application of hair removing cream at least two days prior to the treatments. Only mice that did not show signs of hair re-growth were used.

### Preparation of iron dextran solution

Each 10 mL dose vial containing 500 mg of iron as iron complex with a low molecular weight dextran fraction was diluted in saline to obtain a suitable dose.

### Iron estimation conditions

The atomic absorption spectrophotometer gives results directly in ppm. All instructions for instrument setting, calibration and assay as laid down in the manual were strictly followed.

### Serum tissue iron estimation

For serum preparation, blood was collected from the retro-orbital sinus by heparinized capillary tubes into dry test tubes which were kept in tilted positions for 10 minutes at room temperature and then for an hour at 4°C in order to separate the serum from the cellular clot. To remove cellular contamination from the serum, it was centrifuged for 2-3 minutes at 800  $\times$  g. After collection of blood serum, 0.5 mL of serum was transferred to a glass vial containing 1 mL of digestion mixture (1 part of perchloric acid mixed with 3 parts of conc. nitric acid and left for 3 hours at room temperature). It was left overnight, then the glass vials were heated to near dryness on a hot plate. 5 mL distilled water was added to it after which it was slightly heated up to 40-50°C and then filtered using Whatman filter paper through a Buchner funnel into a test tube. The filtrate was read on ASS. Serum iron concentration was calculated and represented as parts per million or  $\mu\text{g/mL}$  serum. Tissues such as liver and skin were excised, cleaned free of extraneous material and washed immediately with saline (0.9%). In case of skin, the dorsal skin of mice was removed, scraped off with a surgical blade to remove fat and blood vessels and was used for iron estimation. 1.0 g of the tissue was taken in a glass vial and added to 10 mL of concentrated HNO<sub>3</sub>. The samples were left overnight. The next day, the samples were found to be dissolved in the acid to a large extent. They were then dried on a hot plate to near dryness. 5 mL

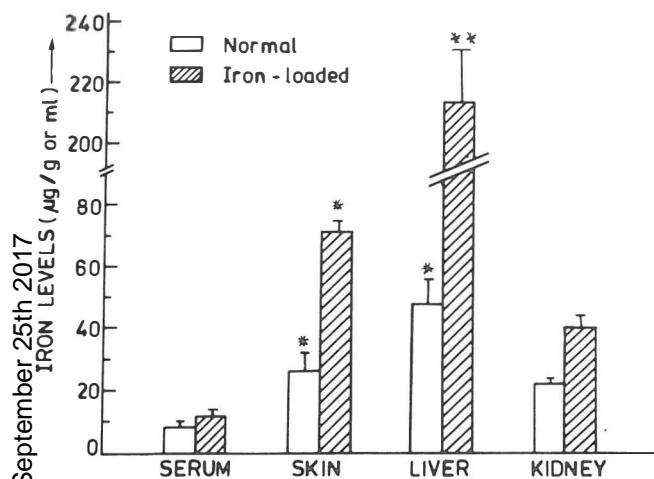


Fig. 1. Effect of iron overload on the tissue iron levels in mice. Each value represents mean±S.E. of six animals. \**p* < 0.001 when compared to saline-treated normal mice. \*\**p* value < 0.05 when compared to saline-treated controls.

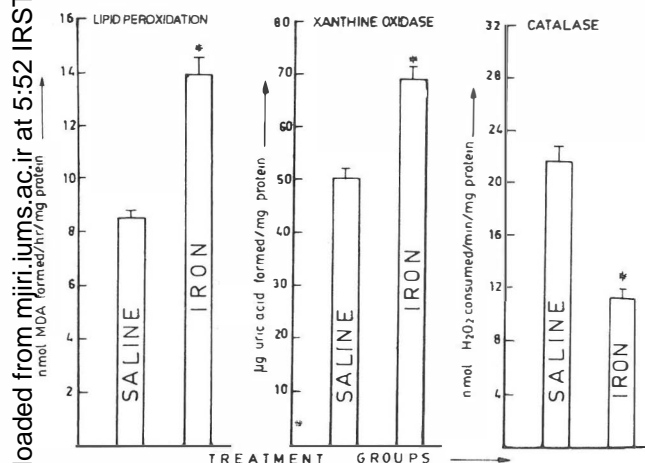


Fig. 2. Effect of iron overload on the enhancement of cutaneous lipid peroxidation, xanthine oxidase and decrease in catalase activity in mice initiated with DMBA. Each value represents mean±S.E. of six animals. \**p* < 0.001 when compared to saline-treated normal mice.

water was added and slightly heated to near boiling. 1 mL of this solution was diluted to 10 mL by adding water. It was read on AAS. Tissue iron concentration was calculated and represented in µg/g tissue.

### Treatment protocol

Treatment protocols, including selection of dose regimens, mode of administration, time period at which animals were sacrificed, etc. will be discussed.

### Treatment of animals and tumor study

For tumorigenesis studies, 40 female Swiss mice were taken and divided into two groups. Group I received

normal saline for a period of two weeks as intramuscular injection (0.1 mL per mouse in hind limb) and served as control. Group II animals received imferon injections intramuscularly for a period of 2 weeks (1 mg iron/0.1 mL saline/mouse/day). Both groups of animals received five topical applications of DMBA (40 µg/0.2 mL acetone/mouse) daily on the dorsal skin 24 hours following the last injection of saline or imferon. No further treatment in any of the two groups of mice was given. The mice were observed weekly for the occurrence of tumors, which were recorded and plotted as function of weeks on test. The data were presented both as number of tumor per mouse as well as percent incidence of tumorigenesis.

### Diagnosis of various skin tumors

The criteria for the diagnosis of various tumors were as described earlier.<sup>20</sup> Papillomas were identified by their cauliflower-like exophytic tumorous growth with a narrow or broad base consisting of a series of connective tissue folds covered by stratified squamous epithelium, usually without cellular atypia. Squamous cell carcinomas are composed of very irregular endophytic epithelial growth with frequent cellular atypia. They are usually very differentiated, with the formation of keratinized layers of horny pearls.

### Experimental protocol

For biochemical studies, two groups of mice (6 mice in each group) were taken. Group I received a single intramuscular injection of saline whereas group II received an intramuscular injection of imferon (5 mg iron/0.2 mL/mouse). 24 hours after the injection of saline/imferon all the animals were killed by cervical dislocation. Their dorsal skin was excised and processed for tissue preparation.

### Tissue preparation for biochemical assays

After the desired time period, control and treated animals were sacrificed by cervical dislocation. The animals were immediately dissected to remove their skins which were washed in ice cold saline (0.85% NaCl). The subcutaneous layer of skin was completely scraped off with a scalpel blade to separate the epidermal layer by keeping the skin sample upside down on a petri dish containing crushed ice. The washed tissue was blotted between the folds of a filter paper and weighed on a metler balance. For biochemical studies a known amount of tissue was taken, minced properly and homogenized in appropriate buffer keeping the samples inside the crushed ice. Tissues were homogenized in a polytron homogenizer (Kinematica A.G.).

### Estimation of tissue iron level

For estimating tissue iron levels in normal and iron

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overloaded animals, the animals were divided in two groups of six mice each. Group I animals received a daily intramuscular injection of saline for fourteen days whereas group II animals received an intramuscular injection of imferon (1 mg Fe/mouse) daily for a period of fourteen days. 24 hours after the last injection of saline or imferon, all animals were killed and their tissues removed and processed for iron estimations. Just before sacrificing, blood was withdrawn from all the animals and kept in separate tubes for the separation of serum in order to estimate iron levels.

### Assay of lipid peroxidation

The assay for microsomal lipid peroxidation was done according to the method of Wright et al.<sup>21</sup> The reaction mixture in a total volume of 1.0 mL contained 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL microsome obtained from the homogenate (10% w/v), 0.2 mL ascorbic acid (100 mM), and 0.02 mL ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for one hour. The reaction was stopped by the addition of 1 mL of TCA (10%). Following addition of 1.0 mL TBA (0.67%), all the tubes were placed in a boiling water bath for a period of twenty minutes. In the end, tubes were shifted to crushed ice bath and then centrifuged at 2500 g for 10 minutes. The amount of malonaldehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm using a spectrophotometer (Milton Roy-21D) against a reagent blank. The results were expressed as nmol MDA formed/hr/gm of tissue at 37°C by using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### Catalase activity

Catalase activity was assayed by the method of Claiborne.<sup>22</sup> Briefly, the assay mixture consisted of 1.95 mL phosphate buffer (0.05 M, pH 7.0), 1.0 mL hydrogen peroxide (0.019 M), and 0.05 mL PMS (10% w/v) in a total volume of 3.0 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol  $\text{H}_2\text{O}_2$  consumed/min/mg of protein.

### Xanthine oxidase (XOD)

XOD catalyzes the conversion of xanthine to uric acid which has a characteristic absorption peak around 290 nm. The following spectrophotometric method based on the modified procedure as described by Striee and Corta<sup>23</sup> is used here. The reaction mixture containing 0.2 mL PMS diluted to 1 mL with buffer was incubated for 5 minutes at 37°C. Reaction was started by adding 0.1 mL xanthine. The reaction mixture was kept at 37°C for 20 minutes. The reaction was terminated by the addition of 0.5 mL ice-cold perchloric acid (40%). After ten minutes, 2.5 mL distilled water was added to the precipitated mixture

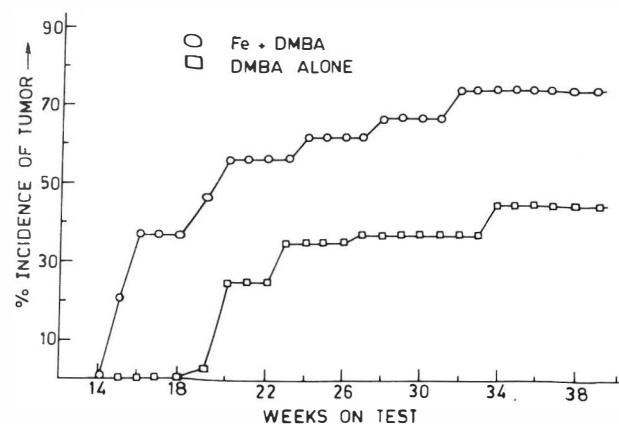


Fig. 3. Percent incidence of tumors in DMBA-treated normal and iron overloaded mice. The data were recorded every week and plotted as a function of weeks on test.

Each value represents % incidence data calculated on the basis of 20 animals.

which was then centrifuged at 4000 rpm for 10 minutes. The clear supernatant was read at 290 nm and results were expressed as uric acid formed per mg protein. 0.360 OD corresponds to 0.2 mg uric acid.

### Estimation of protein

Protein was estimated by the method of Lowry et al.<sup>24</sup> using bovine serum albumin as a standard.

### Statistical analysis

The level of significance between different groups are based on Dunnett's t-test followed by analysis of variance test. The level of statistical significance was chosen at  $p < 0.05$ .

## RESULTS

The effect of iron overload in various organs and serum of mice is shown in Figure 1. Iron overload produces a slight increase in the serum iron whereas tissue iron levels show a marked increase as compared to saline-treated control animals. In skin, iron levels show a nearly three-fold increase whereas, in liver, it was more than four-fold.

The effect of iron overload in mice skin-induced lipid peroxidation, xanthine oxidase and catalase activities in normal and iron overloaded mice skin is given in Figure 2. The iron overloaded animals augmented lipid peroxidation in comparison to saline alone treated animals. Increased xanthine oxidase activity was observed in iron overloaded animals, while catalase activity shows a decrease in iron overloaded animals up to a level of less than 60% as compared to the saline-treated control group.

The effect of iron overload on DMBA-induced

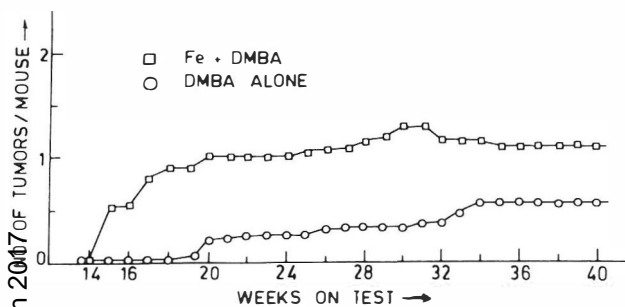


Fig. 4. Effect of iron overload on the yield of tumors developed as a result of DMBA-treatment in mice.

The tumors were counted every week and represented as number of tumors/mouse plotted as a function of weeks on test.

Each value represents mean of number of tumors/mouse calculated on the basis of 20 animals.

Complete carcinogenesis is shown in Figure 3 & 4. Figure 3 shows the percent incidence of tumors plotted against the weeks on test. The first tumor incidence in DMBA only treated animals was observed at week 19. However, the iron overloaded animals receiving a similar topical treatment of DMBA showed the first tumor incidence by week 15. The tumor incidence at week 20 was 25% in the DMBA alone treatment group as compared to 55% in the iron overloaded and DMBA-treated group. The maximum tumor incidence could be observed by week 34 in both the groups (45% in the DMBA alone treatment group against 75% in iron overloaded and DMBA-treated animals). Figure 4 shows the number of tumor per mouse in DMBA alone and DMBA-treatment in iron overload groups. The number of tumors induced in the DMBA alone-treated group was 0.25 per mouse against 1 tumor per mouse in the iron overload group. The maximum number of tumors per mouse at week 30 in the two groups was 0.4 per mouse and 1.25 per mouse, respectively, in DMBA alone and iron overloaded and DMBA-treated animals.

## DISCUSSION

Similar to other polynuclear aromatic hydrocarbons, DMBA is activated by specific enzyme reactions to exert its neoplastic effects. It is metabolized by sequential reactions catalyzed by the cytochrome P-450 IAI-dependent monooxygenase system and epoxide hydrolase to its diol derivative, a compound which can be further metabolized by the two distinct pathways of epoxidation.<sup>25</sup> The stereochemistry of epoxidation by the cytochrome P-450 dependent monooxygenase system and by free radicals (peroxyl radicals) is distinct and produces unique diol epoxides.<sup>26,27</sup> Interestingly, both of these pathways exist in the mammalian skin. The DNA lesions produced as a

result of either enzyme-dependent or free radical-dependent mechanisms are crucial for tumor development. Many of these metabolites may be converted into toxic quinones which oxidatively transfer an electron to produce superoxide anions. Thus, besides being initiators of carcinogenesis, they also produce tumors by exerting a promoting effect. In the present study, the observed decrease in the latency period of tumor induction indicates that iron overload exerts a promoting effect on the DNA lesions produced by DMBA. Since iron enhances skin lipid peroxidation and generates an oxidative stress in the skin, the higher production of free radicals may be responsible for the observed enhancement in the number and incidence of tumors in iron overloaded animals as compared to animals having normal iron levels. In addition, the observed increase in LPO and XOD activities in iron overloaded animals along with the decreased CAT activity in cutaneous tissues are suggestive of the tumor promoting effect of iron overload. The effect of iron overload on the tumor promoting response is evident from the data of the present study where enhanced activity of xanthine oxidase along with lipid peroxidation and diminished catalase activity was observed in iron overloaded animals. This is also supported by the fact that in the present study, increased iron overload-mediated enhancement of oxidant enzyme activities and enhanced iron overload-mediated decrease in the antioxidant enzyme activities were seen.

It therefore seems that iron overload provides similar promoting potential as can be observed with a number of oxidants. This may be because of the production of *in situ* peroxidants by the iron overload in the tissue. Our results of the high cutaneous iron levels also corroborate the tumor data further suggesting an involvement of the iron-mediated biochemical cascade in the development and enhancement of skin tumors. In our previous studies,<sup>28,29</sup> we have shown that *in situ* generated reactive oxygen species as a result of skin porphyrin photosensitization acts as a mild tumor promoting agent. The present data also support our findings that oxidants generated by iron overload may be acting as a modest tumor promoter, thus enhancing the tumor yield in iron overloaded animals. Our data, therefore, show that iron overload enhances DMBA-induced carcinogenesis in skin and suggest that oxidative stress generated by iron overload may be responsible for the observed augmentation of cutaneous tumorigenesis.

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