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Original Article

Experimental data of respiratory and circulatory responses with histopathological changes of spleen to high nitrate ingestion in rabbits

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ABSTRACT

ABSTRACT In India, especially in Rajasthan people drink water containing high level of nitrates and concentration up to 500 mg of nitrate ion per liter is not unusual. The ingested nitrate is converted to nitrite in the digestive system and absorb in blood causing methemoglobinemia. Methaemoglobin is not restricted to infants alone but it is prevalent in higher age groups also. The peak of methaemoglobin is observed at 45-95 mg/liter of nitrate concentration of water. Therefore an experimental study was conducted in 10 rabbits between three and half month to four month of age having weight ranging 1.310 kg to 10720 kg. Five groups A, B, C, and D & E were formed with two rabbits in each group. The control group A was given water orally having 06 mg/liter. Group B to E (experimental groups) were administered water orally having concentration of 100mg/liter, 200mg/liter, 400mg/liter & 500mg/liter of nitrate respectively for 120 days. During experimental period the difference in general behavior of rabbits were noted. After that rabbits were anaesthetized & sacrificed according to guidelines of ICMR and spleens were removed & processed for paraffin sections, hemotoxyllin and eosin staining was done for microscopic observations. During experimental period, the animals were lethargic on 75th day and cyanosis appeared on nails, lips, tongue & sclera on 90th day (mainly in rabbits of group D & E). The respiration rate & heart rate were increased with loss of weight. The microscopic study revealed mild congestion of white pulp. In higher groups, the spleen was showing severe red pulp expansion and infiltration of neutrophils in red pulp which appeared from group D.

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1. Introduction

The majority of Indian population is exposed to nitrate through ground water and dietary sources [1]. Excessive nitrate concentration in drinking water is reported to have caused methaemoglobinemia in infants up to 6 month of age [2,3]. Maximum permissible limit for nitrate ion in drinking water have been set at 50mg/liter by WHO and 45 mg/liter by Bureau of Indian standard (IS-10500) [4-7]. In several developing countries, especially in India, consumption of water containing high nitrate

concentration, at times up to 500mg/liter is not uncommon [8,9]. Rabbits were chosen as the animal because their stomach pH is similar to that of infants (3.05.0) [9,10]. Oxygen is essential for formation of methemoglobin by nitrite. Nitrate are reduced to nitrite by micro flora in the oral cavity & increased consumption of nitrite leads to :- increased production of nitrates, excess nitric oxide generation which has vasodilator effects, enhanced absorption of sodium from intestinal lumen, and increased production of oxygen which will react with other cell constituents possibly causing irreversible damage [11]. There are three stages of interaction between sodium nitrite and blood as :- an induction period, a reactionary period & a terminal period, often prolonged during which the product of reaction, chiefly methemoglobin pass into hematin and other degradation products [12]. The health risk from exposure to nitrate is therefore related not only to their

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concentration in drinking water and food but also condition conducive to their reduction to nitrites [13]. As the above reported data, it was planned to study the toxicological effects of nitrate exposure in drinking water in an appropriate animal study under laboratory conditions.

2. Material and Methods

The study was conducted in the department of Anatomy, S.M.S Medical college and attached group of hospitals, Jaipur, Rajasthan on five groups of 2 rabbits each. The rabbits were used for the study because their stomach pH is similar to infant (pH= 3.0-5.0) [9, 10]. The age of rabbits were three and half to four months & weight varied from 1.310 kg to 1.720 kg. These groups were identified as A,B,C,D & E. Ad libitum quantity of water containing 45,100,200,400 and 500 mg/liter nitrate (in form of NaNO_3) and food soaked in the same water were given to group A to E respectively.

The group consuming 45mg/liter served as a control group. Observations were made during the experimental period of 120 days for the changes in physical activity of the animals on a predesigned performa after every 15 days. After 120 days the animals were sacrificed according to the guidelines of ICMR [11-15] and dissected. The spleens were removed and biopsy was taken from the organ. These tissues were fixed in 10% formalin solution and subjected to histopathological examination.

3. Results and Discussion

Cyanosis was appeared in rabbits of group C, D & E only. In group C, the cyanosis was seen in nails on 105th day. In group D, the cyanosis was observed in nails & sclera on 90th day and on lips it appeared on 105th day. In group E, the cyanosis was observed in nails, sclera & lips on 90th day in both rabbits but in tongue & mucous membrane of oral cavity, the cyanosis was observed in one rabbit only on 105th day. The rabbits of group A did not show lethargy throughout the experimental period. In group B,C & D lethargy appeared on 90th day. The rabbits of group E become lethargic on 60th day. Rabbits of all groups i.e. A to E showed continuous increase in heart rate and respiration rate.

The diarrhea was observed in both the rabbits of group D on 120th day and 90th day in rabbits of group E respectively. These findings are in accordance with the results of Gupta SK et al [13], Comly et al [14], Gupta SK et al [9], Farrant M et al [15] and Greenberg et al [16] which indicates that high nitrate creates problem with oxygen carrying capacity of blood. Increase in heart rate and respiration rate was proportional to the nitrate concentration in drinking water. These findings are similar as those of Kielbase et al [17].

The essential action in the formation of methemoglobin is an oxidation of the ferrous to ferric ion [18]. This action may be brought about in one of the following way [9,11,19]. By direct action of the oxidant or by the action of hydrogen donor in the presence of oxygen or by auto oxidation. In the presence of nitrites, the ferrous ion of hemoglobin gets directly oxidized to ferric state. Normally the methemoglobin is formed is reduced by the following reaction:

$$\text{Hb}+3+\text{Red.Cyt b5} \text{ -----} \rightarrow \text{Hb}+2+\text{Cyt b5}$$

Reduced cytochrome b5 is generated by the enzyme cyt.b5 reductase:

$$\text{Oxy cyt b5} + \text{NADH} \text{ -----} \rightarrow \text{Red cyt b5} + \text{NAD.}$$

Thus the enzyme cyt b5 reductase plays a vital role in counteracting the effect of nitrate ingestion.

No histopathological changes were observed in spleen of the rabbits of group A & Group B. The changes were appeared in group C in the form of mild congestion of white pulp. The changes were more marked as the nitrate concentration increased in the form of red pulp expansion, numerous deposits of brown blood pigments and infiltration of neutrophils in red pulp which appeared in group D & E. These findings are in accordance with Farrant M et al [15] who observed the same findings in spleens.

Figure: 1. Microphotograph of spleen showing normal histology (10X), GROUP-A

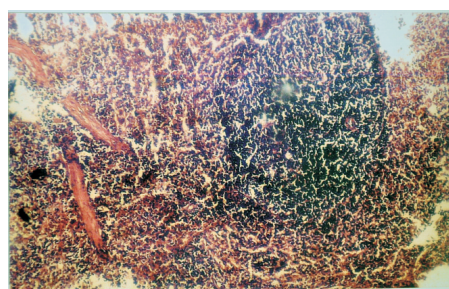


Figure: 2 . Microphotograph of spleen showing chronic congestion of white pulp (10X), GROUP- C.

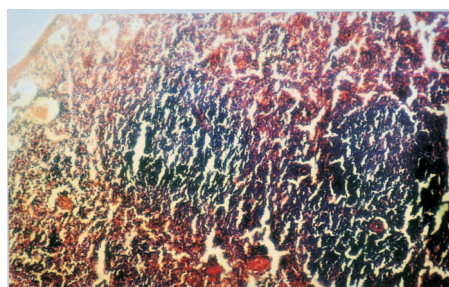


Figure: 3 . Microphotograph of spleen showing chronic congestion of white pulp and expanded red pulp (10X), GROUP- D.

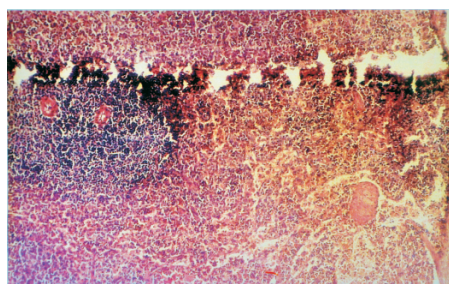


Figure: 4 . Microphotograph of spleen showing severe expansion of the red pulp (10X), GROUP- D.

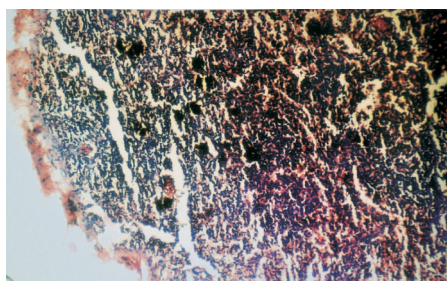
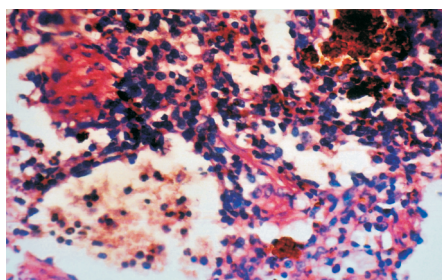


Figure: 5 . Microphotograph of spleen showing severe expansion of the red pulp, Neutrophilic infiltration and collection of hemosiderin (40X), GROUP- E.



| | SPLEEN | CAPSULE | WHITE PULP | RED PULP | TRABECULAE |
|-----------|--------|---------|------------|----------|------------|
| 5 GROUP A | NORMAL | NORMAL | NORMAL | NORMAL | NORMAL |

Table-1 Comparative histopathological changes in spleen of Rabbits in all groups.

| | SPLEEN | CAPSULE | WHITE PULP | RED PULP | TRABECULAE |
|---------------------|---------|---------|------------|------------|------------|
| 5 Groups of Rabbits | GROUP A | NORMAL | NORMAL | NORMAL | NORMAL |
| | GROUP B | NORMAL | NORMAL | NORMAL | NORMAL |
| | GROUP C | NORMAL | + | NORMAL | NORMAL |
| | GROUP D | NORMAL | ++ | X\$ | NORMAL |
| | GROUP E | NORMAL | ++++ | XXX \$\$\$ | NORMAL |

X = Neutrophilic infiltration

\$ = Red pulp expansion

(+) = Mild congestion

(++) = Moderate congestion,

(+++)= Severe congestion.

NORMAL= Normal histology

TABLE -2 Comparison in Physical activity of Rabbits in all groups during experimental period of 120 Days

| | FIVE GROUPS WITH ANIMAL | | LETHARGY | | | | | | | | | | STOOL WITH MUCOUS AND DIARRHOEA | | | | | | | | | | |
|--------------------------------|-------------------------|---|----------|---|-----|---|-----|---|-----|---|-----|---|---------------------------------|---|-----|---|-----|---|-----|----|-----|----|----|
| | | | GPA | | GPB | | GPC | | GPD | | GPE | | GPA | | GPB | | GPC | | GPD | | GPE | | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | |
| 1st Day | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Days of observation (120 Days) | 15th Day | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 30 th Day | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 45th Day | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| | 60th Day | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | + | + |
| | 75th Day | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | + |
| | 90th Day | - | - | + | - | - | + | + | + | + | + | - | - | - | - | - | - | - | + | + | - | • | + |
| | 105th Day | - | - | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - | + | + | - | +• | +• |
| | 120th Day | - | + | + | - | + | + | + | + | + | + | - | - | - | - | - | - | - | +• | +• | - | +• | +• |

GP = Groups

+ = Appearance of symptoms

- = No symptoms was observed

• = Appearance of Diarrohea

TABLE -3 Comparison in Physical activity of Rabbits in all groups during experimental period of 120 Days

| | FIVE GROUPS WITH ANIMAL | | LETHARGY | | | | | | STOOL WITH MUCOUS AND DIARRHOEA | | | | | | | | | | | | | |
|-----------|-------------------------|-----|----------|-----|-----|-----|-----|-----|---------------------------------|-----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|
| | | | GPA | | GPB | | GPC | | GPD | | GPE | | GPA | | GPB | | GPC | | GPD | | GPE | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 1st Day | 140 | 144 | 140 | 142 | 142 | 140 | 142 | 140 | 142 | 140 | 56 | 58 | 56 | 58 | 56 | 56 | 56 | 58 | 56 | 58 | 56 | 56 |
| 15th Day | ↑ | - | - | ↑ | - | ↑ | ↑ | - | - | - | ↑ | - | - | - | - | ↑ | - | - | ↑ | - | - | |
| 30th Day | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | - | ↑ | - | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - |
| 45th Day | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | - | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | ↑ |
| 60th Day | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | - | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| 75th Day | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | - | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| 90th Day | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | - | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| 105th Day | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | - | ↑ | - | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| 120th Day | 158 | 158 | 152 | 158 | 166 | 168 | 192 | 198 | 216 | 216 | 60 | 60 | 58 | 60 | 64 | 60 | 64 | 64 | 64 | 64 | 72 | 84 |

GP = Groups

↑ = Increase in Parameter

↓ = Decrease in Parameter

- = No change was observed

4. Conclusion

The results of present study proved strong interdependence between high nitrate concentration and changes in physical activities with histopathological changes of spleen in rabbits. The degree of damage was more pronounced as nitrate concentration increased in drinking water. A possible cause could be the reverse of cyt.b5 reductase activity and its adaptation with increasing water nitrate concentration to compensate methaemoglobinemia. While an isolated study can be not extrapolated to humans it highlights the need for conducting further studies in population consuming nitrate rich water.

The nitrate problem has not been taken up seriously in our country. It is expected that the finding of this study will draw attention of decision maker to take note of this serious problem and take adequate step to ensure that safe drinking water is available to public.

Abbreviations

1. Cyt b5 ----- Cytochrome b5
2. Hb+2 ----- Ferrous state of hemoglobin
3. Hb+3 ----- Ferric state of hemoglobin
4. ICMR ----- Indian Council Of Medical Research
5. Kg ----- Kilogram
6. mg ----- Milligram
7. NAD ----- Nicotinamide adenine dinucleotide
8. NADH ----- Nicotinamide adenine dinucleotide hydrogenase
9. pH ----- Negative logarithm of hydrogen ion
10. Red cyt b5 ----- Rduced cytochrome b5
11. Oxy cytb5 ----- Oxidized cytochrome b5
12. WHO ----- World Health Organization

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