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Original Article

To Study the Effect of Vitamin D and E on Sodium-Fluoride-induced Toxicity in Reproductive Functions of Male Rabbits

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ABSTRACT

Objectives: Fluorosis is an endemic problem in many countries of world. This study was designed to see the effect of fluoride on the reproductive system and to see the role if any of vitamin D or E supplementation on it. **Materials and Methods:** Sixty rabbits were divided into six equal groups. Group I was fed on standard diet, Group II vehicle treated control, Group III on sodium fluoride (NaF) 20 mg/kg body weight, Group IV on NaF + Vitamin D, Group V on NaF + vitamin E, and Group VI on NaF + vitamin D + vitamin E. **Results:** In Group III (fed on sodium fluoride) significant decrease in sperm count ($P<0.001$), motility ($P<0.001$), progressive motility ($P<0.01$), and epididymal weight ($P<0.05$) compared to control was seen that was also evident on testicular histology. With vitamin D supplementation, there was a significant improvement in the sperm count ($P<0.001$), motility ($P<0.01$), and progressive motility ($P<0.05$) but remained significantly lower than the control values. With vitamin E supplementation there was significant improvement in the sperm count near normal. With vitamin D and E combined supplementation there was significant improvement in both sperm count and motility near to normal. **Conclusions:** We conclude that combined vitamin D and E treatment showed a significant improvement in reproductive functions affected by fluoride.

Key words: Fluoride, histology, rabbits, sperm, vitamin D, vitamin E

INTRODUCTION

Fluorosis is an endemic public health problem in many nations around the world. The World Health Organization (WHO) guideline is that 1.5 ppm of fluoride is the desirable upper limit in drinking water and the recommended levels are 0.5 to 0.8 mg/L.[1] Extensive data on skeletal fluorosis are available. However, the effect of fluoride on the structure and metabolism of several soft tissues has been reported recently and convincing evidence from fluorosis patient is now available to demonstrate the damage or involvement of human spermatozoa.[2] Chinoy and Sequeira reported that sodium fluoride treatment in mice caused alteration in histology of reproductive organs, morphology of sperm, and induced biochemical changes.[3,4] In fluoride-treated male rats there was decrease in sperm motility.[5] In an Indian study, infertility was reported among married men in a highly endemic area with fluoride concentration up to 38.5 mg/L.[6] Sodium fluoride treatment (20 mg/kg/day for 29 days) results a significant diminution of relative wet weight of the testis, prostate, and seminal vesicle, without alteration in the body weight.[7] Susheela and Kumar have shown that chronic fluoride toxicity caused regression of seminiferous tubule and cessation of the spermatogenesis in the rabbit.[8] Fluoride treatment leads to oxidative stress as indicated by an increased level of conjugated dienes in the testis, epididymis, and epididymal sperm with respect to control.[7] Vitamin D (vit D) hormone plays an important role in reproduction.[9-11] Sood *et al*.

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suggested that deficiency of vitamin D produces retardation of spermatogenesis due to disturbances in sertoli cell functions, and these changes are reversible and can be corrected by supplementing an optimal dose of vitamin D.[12] Vitamin E (vit E) is believed to exert its protective effect at the cellular – molecular level, primarily through destruction of cell damaging free radical oxygen species.[13] Chinoy and Sharma reported that the supplementation of vitamin D and E during the withdrawal period of sodium-fluoride-treated mice was found to be very beneficial in recovery of all sodium-fluoride-induced effects on reproductive functions and fertility. The extent of recovery was more pronounced with vitamin E as compared to vitamin D and was most significant with the combined treatment. However, not many studies are available to show the effect of vitamin D and E in fluorosis.[14]

Considering that fluorosis is a public health issue and the very fact that the fluoride exposure has a definite effect on reproduction. The following study was planned to observe the effect of fluoride on the sperm count, motility, and histology of testis and to study the ameliorative effect, if any of vitamin D and E either alone or in combination.

MATERIALS AND METHODS

The study was conducted in the Department of Physiology in collaboration with Department of Pathology after approval by Institutional Animal Ethical Committee. Sixty healthy, adult New Zealand white male rabbits were used for the study. All the rabbits were of same age group, with weight range of 1.5 – 2.5 kg. The rabbits were housed in a well-ventilated animal house and caged separately, at a temperature of 29°C and 32°C and exposed to 10 to 12 h of daylight. They received food and water ad libitum. The rabbits were divided into six equal groups of 10 each. Sodium fluoride (analytic reagent) was given using a feeding tube attached to a hypodermic needle in the dose of 20 mg/kg body weight/day.[15] Doses of vitamin D and vitamin E were 1000 I. U./kg body weight/day and 100 mg/kg body weight/day respectively in addition to normal Diet.[16,17] These doses were based on previous studies.

- Group I: (untreated control) rabbits were maintained on standard diet and water ad libitum.
- Group II: (vehicle treated control) rabbits were fed olive oil in which vitamins D and E are dissolved.
- Group III: Rabbits were given sodium fluoride (NaF) for 30 days and were sacrificed on the 31st day.
- Group IV: Rabbits were given sodium fluoride (NaF) with vitamin D for 30 days and sacrificed on the 31st day.
- Group V: Rabbits were given sodium fluoride (NaF) with vitamin E for 30 days and sacrificed on the 31st day.
- Group VI: Rabbits were given sodium fluoride (NaF) with vitamin D and vitamin E for 30 days and sacrificed on the 31st day.

The control and the test group were anaesthetized with intravenous injection of Urethane (0.5–1.5 g/kg of body weight). Incision was taken on the scrotum of the rabbit and the testis and epididymis was carefully exposed, removed, and were subjected to the following physiological and histopathological studies.

Physiological studies

Sperm count

From each separated epididymis, the cauda part was removed and placed in a beaker containing 10 mL diluting solution (sodium bicarbonate 5 g and formalin neutral 1 mL in 100 mL of distilled water). Each section was quickly macerated with a pair of sharp scissors and left for a few minutes to liberate its spermatozoa into the solution. Sperm count was done under the microscope using a new improved Neubauer Heamocytometer and the sperm count was calculated per epididymis.

Sperm motility and progressive motility

Semen drop was placed on the slide and two drops of warm 2.9% sodium citrate was added. The slide was covered with a cover slip and examined under the microscope using ×40 objective for sperm motility and progressive motility.[18]

Histopathology

The *weight* of testis and epididymis of test Groups (III to VI) was taken and compared with the control group.

Histopathology of testis

Testis was removed after being freed from surrounding tissue. The tissue was kept in a 10% neutral formalin solution for fixation. After 1 week, the tissue was washed for 24 h under running tap water, then dehydrated through ascending grades of alcohol, cleared in xylene and embedded and blocked in paraffin. Sections 4–5 μm thickness was taken and stained with hematoxylin and eosin, and was examined under the microscope.

RESULTS

There was no statistically significant difference of age and weight of animals between different groups (I–VI). Also there was no significant difference between Group II (vehicle-treated group) and Group I (control).

Sperm count

Table 1 shows that the sperm count in the cauda epididymis

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Table 1: Comparison between different reproductive parameters of control, vehicle treated control and test groups (ANOVA)

Parameters	Sperm count $\times 10^6$/ epididymis	Sperm motility (%)	Progressive motility of sperm (%)	Testicular weight (gm)	Epididymal weight (gm)
Group I (control)	164.98 ± 9.58 b***	72.1 ± 3.28 b***	58.6 ± 6.02 b**	3.22 ± 0.46	0.63 ± 0.21 b*
Group II (vehicle treated control)	160.45 ± 9.09	71.9 ± 4.22	57.7 ± 6.09	3.13 ± 0.56	0.64 ± 0.19
Group III (fed on NaF)	121.45 ± 13.01 a***	59.1 ± 10.28 a***	47.1 ± 11.44 a**	2.86 ± 0.37	0.46 ± 0.06 a*
Group IV (NaF+Vit D)	145.57 ± 6.13 a***, b**	62.7 ± 3.94 a**	48.4 ± 5.75 a*	2.94 ± 0.25	0.52 ± 0.05
Group V (NaF+Vit E)	153.45 ± 9.13 b***	65.1 ± 6.75	51.5 ± 6.62	3 ± 0.29	0.51 ± 0.05
Group VI (NaF+Vit DandE)	159.35 ± 3.07 b***	68.8 ± 3.08 b**	56.4 ± 4.52	3.11 ± 0.36	0.62 ± 0.04

a: Comparison with Group I (control), b: Comparison with Group III *P<0.05, **P<0.01, ***P<0.001 (n=10, all values in mean ± SD)

of Group III NaF-treated mice was significantly (P<0.001) reduced compared to control Group I. In Group IV (NaF with Vitamin D) and V (NaF with vitamin E) significant (P<0.01 and P<0.001, respectively) improvement was seen that was again more with vitamin E. In Group VI the recovery was most significant (P<0.001) that was near normal showing no significant (P<0.001) difference with control Group I.

Sperm motility and progressive motility

Group III showed a significant decrease in sperm motility (P<0.001) and progressive motility (P<0.01) as compared to the control Group I. No significant improvement was seen in Group IV and V where vitamin D or E alone was given but a significant improvement (P<0.01) was seen in sperm motility of Group VI where combined treatment with vitamin D and E was given [Table 1].

Epididymal and testicular weights

The weight of epididymis in Group III declined significantly (P<0.05) compared to the control group. In Group IV and V, the recovery was less and the difference was not significant as compared to control, whereas significant (P<0.001) recovery near normal was obtained in Group VI where both the vitamins (D and E) were given simultaneously. However, weights of testis were not affected by different treatments [Table 1].

Histopathology of testis

Group I – histology of testis showed normal spermatogenesis with different stages of differentiation and maturation. Spermatozoa were in groups attached to the inner aspect of the lumen of the seminiferous tubules [Figure 1].

Group II – shows normal spermatogenesis with different stages of differentiation and maturation like that of control group (group I) [Figure 2].

Group III – there was lack of differentiation and maturation of spermatocytes and there was marked infiltration in the interstitial area of seminiferous tubules. No mature spermatozoa were seen in the lumens of the seminiferous tubules [Figure 3].

Group IV – shows improvement in the spermatogenic activity in Group V, a small number of mature spermatozoa was also seen in the lumen of tubules as compared to the group fed on fluoride for 30 days but the recovery was not up to the control [Figure 4].

Group V – a greater improvement was seen when vitamin E is given along with sodium fluoride for 30 days. There was normal spermatogenesis in many of the seminiferous tubules [Figure 5].

Group VI – marked improvement near to the normal control group was seen when combined vitamin D and E were given along with sodium fluoride for 30 days [Figure 6].

DISCUSSION

Sperm count

On exposure to 20 mg/kg body weight sodium fluoride for 30 days there was highly significant (P<0.001) decrease in the epididymal sperm count as compared to the control group in this study.

Our observations were similar with studies done by other workers who reported that there was a decline in sperm count as compared to control in rats exposed to sodium fluoride 10 mg/kg body weight for 50 days,[19] or with 10 mg/kg fluoride for 30 days in male mice.[14] Effect of fluoride toxicity on spermatogenesis could be because of that fluoride reduces testosterone levels by diminishing positive signals for its production and by reducing testicular zinc levels that impairs the angiotensin converting enzyme (ACE) activity and hence causes inhibition of spermatogenesis.[20] Apart from direct effect fluoride inhibits androgen receptor (AR) mRNA expression in sertoli cells and cause decrease in AR through which testosterone acts.[21] Other factor responsible for the arrest of spermatogenesis might be the lack of available proteins necessary for cell division, growth, and differentiation of germ cells.[19] Other mechanism may be increased levels of oxidants that damages DNA.[22]

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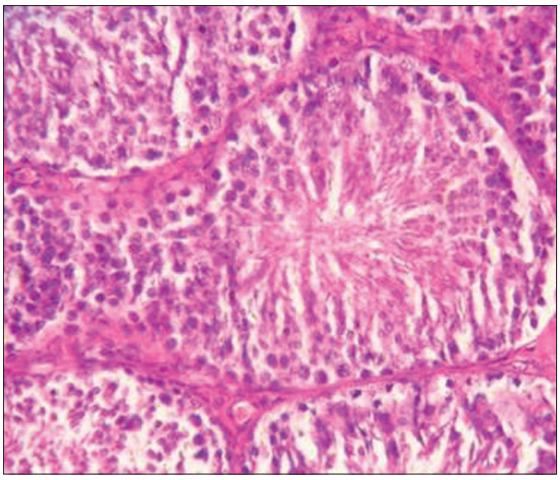


Figure 1: Testicular histology of control Group I (Fed on standard diet and water ad libitum)

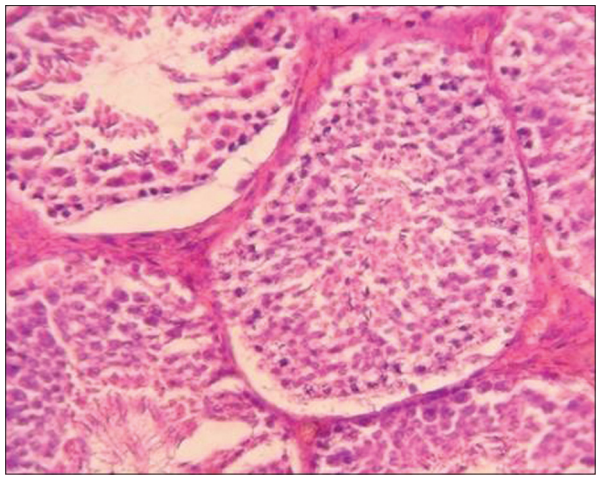


Figure 2: Testicular histology of vehicle treated control Group II

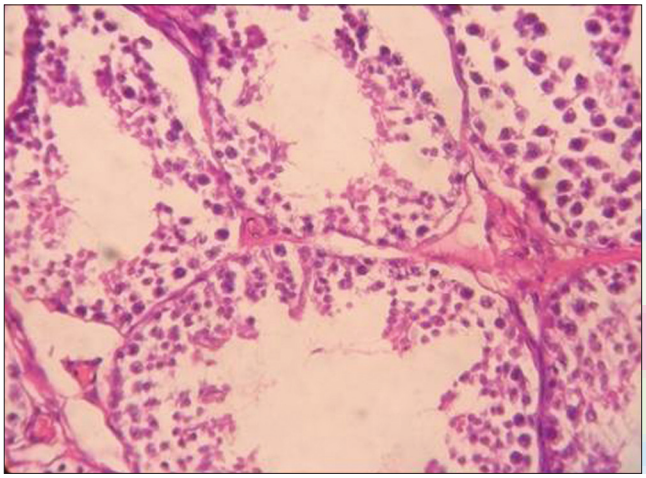


Figure 3: Testicular histology of Group III (fed on NaF)

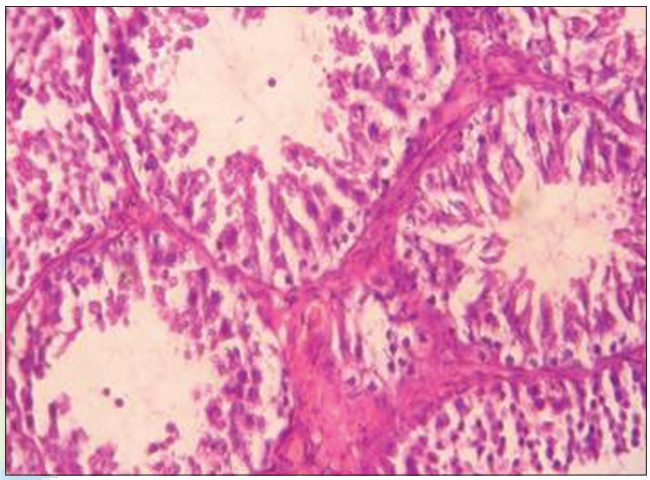


Figure 4: Testicular histology of Group IV (fed on NaF+vitamin D)

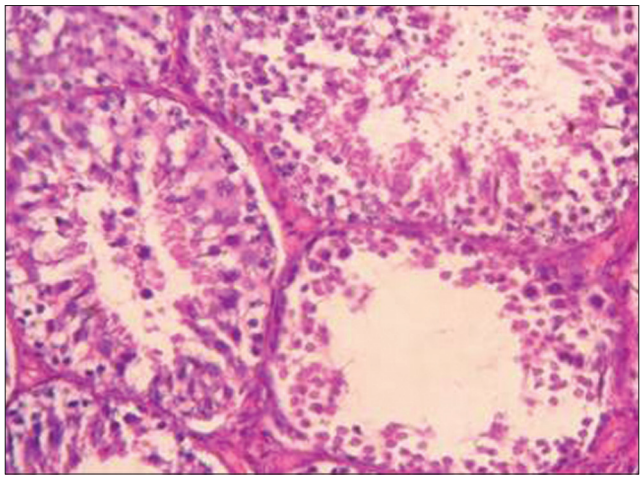


Figure 5: Testicular histology of Group V (fed on NaF+vitamin E)

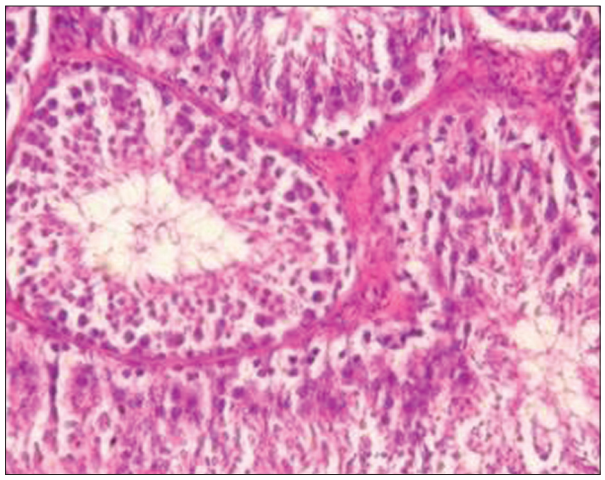


Figure 6: Testicular histology of Group VI (fed on NaF+vitamin D+vitamin E)

This study also showed a significant ($P<0.01$) improvement in the sperm count when fluoride was combined with vitamin D compared to NaF-treated Group III. In an another study, Sood *et al*. from same laboratory reported a significant decrease in sperm count of vitamin D deficient rats as compared to the control and injection of vitamin D improved sperm count after one month. Possible involvement of vitamin D in testicular function

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was suggested by the observation of receptor site for ($1,25[OH]_2 D_3$) in both the seminiferous tubules as well as interstitial tissue in the testis of adult rats and also in human testis also an increase in receptor level was correlated with testicular maturity.[23] Also vitamin D helps in absorption of calcium, which is an ideal antidote for fluoride due to its binding to form insoluble complex calcium fluoride. Moreover, Ca^{++} helps in activation of some enzymes in sperm.[19]

On feeding vitamin E along with NaF there was a highly significant ($P<0.001$) improvement in sperm count compared to group III fed on NaF only. Similar findings were reported earlier in mice.[14] Fluoride has dense negative charge and hence binds with various antioxidants and enzymes, thus reduces oxidative stress in testis.[22] At the cellular level, vitamin E is believed to exert its protective effect primarily through destruction of cell damaging free radical oxygen species produced by fluoride. Hence, vitamin E that is well-known antioxidant ameliorates fluoride induced toxicity due to its antioxidant and detoxification properties.[7]

A highly significant improvement ($P<0.001$) in sperm count was seen in Group VI fed on combined vitamin D and E with NaF compared to Group III (fed on NaF). Similar findings were given by Chinoy and Sharma who reported a significant improvement in the mice sperm count in a group fed on combined vitamin D and E along with fluoride for 30 days as compared to group fed on fluoride 10 mg/kg for 30 days.[14] In this study, there was 72.7% improvement in sperm count while in our study there was only 37.14% improvement that might be because of dose and species variations.

Sperm motility and progressive motility

In this study, there were a significant decrease in sperm motility ($P<0.001$) and progressive motility ($P<0.01$) in group fed with fluoride 20 mg/kg for 30 days as compared to control. Huang *et al*. reported a significant reduction in sperm motility of mice fed on 100, 200, and 300 mg NaF/L for 8 weeks as compared to the control group.[22] Similarly, another study showed a significant ($P<0.001$) decrease on exposure of 10 mg/kg body weight of fluoride for 30 days in mice.[14] Effect of fluoride (30 mM) on the metabolism and motility of ejaculated bull sperm *in vitro* had also been demonstrated, the sperms became immotile within 2 min at 30°C. Similarly, human spermatozoa lost their motility *in vitro* in the presence of 250 mM Fluoride within 20 min of exposure.[19]

The mechanism by which fluoride affects sperm motility has not been clearly elucidated. However, it has been postulated that fluoride could act directly on motile apparatus without affecting other metabolic systems also fluoride binds with cofactors like Mg, Ca, Zn, and Se thus inhibits glycolysis, respiration and motility of sperms. Thus, there could be decline in fructose level due to alteration in carbohydrate metabolism after fluoride treatment.[19,24] Another reason for decreased sperm motility was decreased level of androgen carrier proteins involved in sperm motility.[25]

In our study, no significant improvement was seen in sperm motility and progressive motility of group IV fed on vitamin D with NaF comparison to group III fed on NaF. Chinoy and Sharma reported that in group fed on vitamin D during the withdrawal period of 30 days after exposure to 10 mg/kg body weight NaF for the same period a statistically significant improvement in sperm motility was seen compared to control.[14] The reason of nonsignificant improvement in our study may be because of high dosage of fluoride and species variation.

This study showed a slight improvement but not statistically significant in sperm motility and progressive motility in Group V fed on vitamin E with NaF in comparison to Group III fed on NaF. Earlier another study also reported a similar improvement in sperm motility.[14] At the cellular level, vit E is believed to exert its protective effect primarily through destruction of cell damaging free radical oxygen species produced by fluoride. Hence, vitamin E that is well-known antioxidant ameliorates fluoride-induced toxicity due to its antioxidant and detoxification properties.[7]

Significant ($P<0.01$) improvement in sperm motility was seen in Group VI (fed on fluoride with Vitamin D and E with NaF for 30 days) in comparison to Group III. Similar findings were given by Chinoy and Sharma who reported a significant improvement in mice sperm motility in a group fed on combined vitamin D and E along with fluoride for 30 days as compared to group fed on fluoride 10 mg/kg for 30 days.[14]

Organ weights

In our study, in Group III there was a significant ($P<0.05$) decrease in epididymal weight as compared to control while there was no significant decrease in testicular weight. Similarly in another study rabbits fed on fluoride (10 mg/kg for 23 months) were having a significant decrease in epididymal weight,[26] also the weight of cauda epididymis (9.0 ± 0.5 mg) in fluoride-treated (10 mg/kg for 30 days) mice declined significantly ($P<0.01$) compared to control Groups.[14] However, no significant difference was observed in mean testicular weights of rats fed on 100 and 300 ppm fluoride for 12 weeks when compared with control.[27] Our findings differed with Ghosh *et al*.. who reported an increase in relative testis weight of rats as compared to control with fluoride treatment (20 mg/kg for 29 days).[7]

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Testicular histology

There was lack of differentiation and maturation of spermatocytes with marked infiltration in the interstitial area of seminiferous tubules and no mature spermatozoa were seen in the lumens of the seminiferous tubules in Group III (fluoride 20 mg/kg for 30 days) as compared to normal spermatogenesis showing various stages of differentiation and maturation with spermatozoa attached in groups to the inner aspect of the lumen of the seminiferous tubules in the control group.

Our results were similar to earlier studies which showed that 30 days of treatment with sodium fluoride (10 mg kg body weight) to mice resulted in sloughing off of the spermatogenic cells in the luminal regions of seminiferous tubules of the testis leading to disorganization of their epithelium that caused a complete absence of spermatogenesis in the testis.[25] Opposite to this Sprando *et al*. showed that rats fed on 250 ppm fluoride for 10 weeks showed no distinguishable change in testicular histology from their control group.[20]

Histopathology of testis supported the improvement in the spermatogenic activity in Group IV, V, and VI compared to the Group III (fed on fluoride for 30 days). The extent of recovery was more pronounced with Group V fed on vitamin E as compared to Group IV fed on vitamin D and was most significant with the combined treatment.

CONCLUSION

Therefore we concluded that combined vitamin D and E had a better prospective in ameliorating fluoride induced reproductive toxicity over the alone D or E treatment.

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