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Assays of Embryonic Death in Normal Pregnant Mice Injected at Their Different Days of Gestation with Spores of Two Species of Fungi

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The lethal test in mice by mating male parents treated with odd chemicales and living mutagens (Manna 1985) to normal virgin females has been a standard practice. Originally the method was devised independently and simultaneously by Snell (1935) and Hertwig (1935) and it was extended and elaborated by others (Rohrborn 1970, Soares 1972, Manna 1985). On the other hand, it was found in our laboratory that normal pregnant mice which were injected intraperitoneally with the bacterium, *Xanthobacter autotrophicus* (Manna and Sadhukhan 1991) or the antibiotic, Ampicillin (Manna and Roy 1993) at their different days of pregnancy and the effect was assessed at their 15th day of gestation by vivisection, they caused higher frequency of embryonic deaths as compared to the parallel controls. With a view to verifying if similar effect could also be produced by the application of some other group of microbes, pregnant mice at their different days of gestation were injected separately with spores of the fungi, *Helminthosporium oryzae* and *Aspergillus niger* and the results have been presented here. The mutagenic potential of the spores of these two species of fungi in experimentally treated mice was reported elsewhere by us deploying a battery of tests (Manna and Kundu 1991, Kundu and Manna 1989, 1992).

Materials and methods

About 12 week old laboratory bred normal male and female Swiss albino mice, *Mus musculus* were used as experimental model. Three females to one male together were caged in several sets and the successful mating was ascertained every day by the appearance of vaginal plug which was counted as the first day of conception. Such normal pregnant females at their 4th, 8th and 12th day of gestation were individually injected intraperitoneally with 8×10^6 spores per ml in distilled water of the pathogenic fungus of paddy, *Helminthosporium oryzae* and the pathogenic fungus, *Aspergillus niger* causative agent for aspergillosis and otomycoses in man, at the rate of 1 ml per 100 g body weight of individual specimen. Parallelly mice injected with sterile PDA (potato-dextrose-agar) medium at the same rate served as controls. On the 15th day of gestation, individual control and spores of *H. oryzae* and *A. niger* injected pregnant females were vivisected separately to determine the number of dead among total implants in each series.

Results

Altogether 15 normally pregnant mice injected with sterile PDA medium using 5 each at their 4th, 8th and 12th day of gestation as controls had only one dead embryo out of total 41 implants of five pregnant females injected on their 8th day of pregnancy rendering the frequency of dead implant of 2.4% while in the combined data of 123 implants it was 0.8%

(Table 1). On the other hand, the treatment of spores of *H. oryzae* and *A. niger* to different sets of normal pregnant mice at their 4th, 8th and 12th day of gestation induced high frequency of embryonic deaths of different nature when their uteri were vivisected at the 15th day of gestation (Figs. 1–4). Unlike symmetrical pattern of implantations on two sides of the uteri of control pregnant females, in spores of *H. oryzae* (Figs. 1, 2) and *A. niger* (Figs. 3, 4) treated series, the embryos in some instances appeared somewhat asymmetrically implanted having uneven distribution on two sides of uteri (Figs. 1, 4) while in others it was symmetrical (Figs. 2, 3). The embryonic deaths appeared generally in three forms as (i) a gap in the uterus between other developing embryos leaving practically no trace of implantation possibly due to the complete absorption (Figs. 1a, 2a, 4a), (ii) a scar or reduced lump at the place of implantation representing possibly that the death took place a little later than earlier one (Fig. 1b) and (iii) the size of the implant was apparently normal (Fig. 3c) but on removing the foetal

Table 1. Frequency of dead embryos assessed on 15th day of gestation in normal pregnant females treated (Tr) at their 4th, 8th and 12th day of pregnancy separately with spores of *H. oryzae* and *A. niger* against sterile PDA medium injected controls (cont)

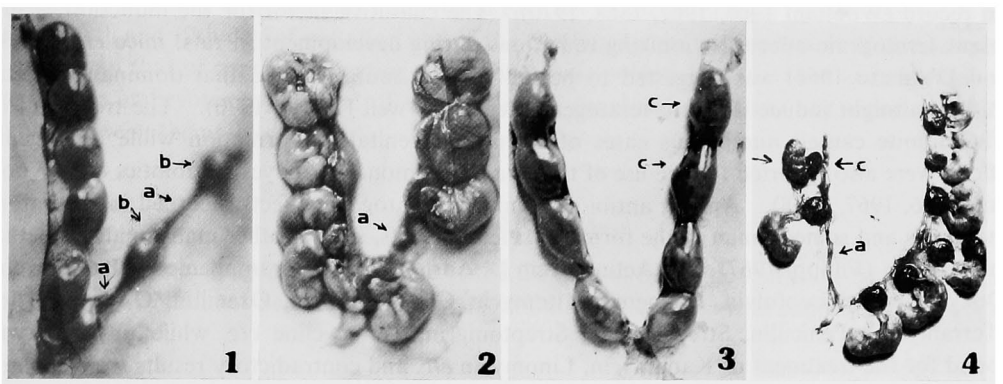
Pregnancy	Series	Mother	Total			Average per mother			% net increase	Tr *vs* Co Tau test
			Implant	Alive	Dead	Implant	Alive	Dead		
A. *Spores of Helminthosporium oryzae treated series*:										
4th day	Tr	5	36	27	9	7.2	5.4	1.8	25.0	P<0.001
	Cont	5	42	42	0	8.2	8.2	0		
8th day	Tr	5	38	30	8	7.6	6.0	1.6	18.6	P<0.01
	Cont	5	41	40	1	8.2	8.0	0.2		
12th day	Tr	5	36	31	5	7.2	6.2	1.0	13.9	P<0.01
	Cont	5	40	40	0	8.0	8.0	0		
Combined	Tr	15	110	88	22	7.3	5.8	1.5	19.2	P<0.001
	Cont	15	123	122	1	8.2	8.1	0.1		
B. *Spores of Aspergillus niger treated series*:										
4th day	Tr	5	39	29	10	7.8	5.8	2.0	25.6	P<0.001
	Cont	5	42	42	0	8.2	8.2	0		
8th day	Tr	5	40	32	8	8.0	6.4	1.6	17.6	P<0.01
	Cont	5	41	40	1	8.2	8.0	0.2		
12th day	Tr	5	34	27	7	6.4	5.4	1.4	20.5	P<0.01
	Cont	5	40	40	0	8.0	8.0	0		
Combined	Tr	15	113	88	25	7.5	5.8	1.7	21.3	P<0.001
	Cont	15	123	122	1	8.2	8.1	0.1		

Tr, Treatment; Cont, control.

membrane some embryos were found dead revealed by various signs like impaired placental connection, retarded or deformed growth (Fig. 4c) as compared to other normally growing ones (Fig. 4), stoppage of heart beat *etc.* Some of the dead embryos outwardly showed blackish texture of membrane, accumulation of fluid *etc.* which were more clearly followed on removing the membrane carefully (Figs. 2, 4). Therefore, the death of the embryos took place at different times after the treatment of spores of *H. oryzae* (Figs. 1, 2) and *A. niger* (Figs. 3, 4) to normal pregnant mice at their different days of gestation. However, for quantitative analysis, all the types were put under only one catagory as dead implants (Table 1). An analysis of the data (Table 1) revealed that the treatment of spores of *H. oryzae* and *A. niger* had in each case a little lower average implantation than that of control assessed at 4th, 8th and 12th day of gestation but the data of three intervals were combined to have a larger sample (Table 1). In the combined data of three intervals, the average number of implants was 7.3 in *H. oryzae* and 7.5 in *A. niger* treated against that of 8.2 per mother of the control series. Therefore, the

average number of implants per mother for the treatment of spores of two species of fungi was not very strikingly different. Further as compared to controls, it was lower by 0.9 in *H. oryzae* and 0.7 in *A. niger* treated series. The occurrence of the lower number of average implants per mother in treated series might be suggested due to the very early death of some implants and subsequently their complete absorption leaving no trace of them on exposing the uteri at the 15th day of gestation for assessment of dead implant in each specimen.

The data (Table 1) further revealed that the frequency of dead implants for the treatment of spores of *H. oryzae* and *A. niger* to different sets of normal pregnant mice at their 4th, 8th and 12th day of gestation was significantly higher than that of control in each case which was maximum for the treatment of spores at 4th day of gestation ($P<0.001$) than that of 8th and 12th day of gestation ($P<0.01$). The frequency of embryonic lethal effect was found to be reduced gradually from early (4th day) to later days of gestation (8th and 12th day) for the treatment of spores of *H. oryzae* but it was not found for the treatment of spores of *A. niger* on the same days of gestation (Table 1). The combined data of the treatment of spores at three intervals of gestation to normal pregnant mice revealed the net increase of dead embryos over control



Figs. 1–4. Some types of embryotoxic effects described in text indicated by arrow as (a) gap, (b) scar, (c) unexposed and exposed dead embryo in the vivisected uteri of normal pregnant mice treated at their 4th, 8th and 12th day of gestation with spores of the fungi, *Helminthosporium oryzae* (Figs. 1 and 2) and *Aspergillus niger* (Figs. 3 and 4).

was 19.2% in *H. oryzae* and 21.3% in *A. niger* treated series and the difference in frequency in each case was statistically highly significant ($P<0.001$) leaving no doubt that the treatment of spores of each species of fungus induced very high frequency of embryonic death even if the data of treatment of spores at each interval of gestation was considered to be somewhat limited.

With a view to comparing the relative sensitivity, the data of embryonic lethal effect at each of 4th, 8th and 12th day of treatment and the combined data of three intervals of *H. oryzae* and *A. niger* treated series were subjected to normal 'Tou' test. The value of 'Tou' obtained was 0.06 ($P>0.05$) for 4th day treatment and 1.25 ($P>0.05$) for 8th day treatment indicating that there was no difference between the embryotoxic effects of the spores of *H. oryzae* and *A. niger* while the treatment made on 12th day of gestation yielded the value of 8.25 ($P<0.001$) and also in the combined data of three intervals the value was 10.5 ($P<0.001$) indicating the embryotoxic effect of the spores was differential. It was somewhat higher in *A. niger* than that of *H. oryzae* except at 4th day of treatment.

The similar type of embryotoxic effects tested on normal pregnant mice treated with the log culture of the bacterium, *Xanthobacter autotrophicus* (=*flavus*) at the 2nd, 4th, 6th and 12th day of gestation yielded significantly high frequencies of embryonic deaths than that of control

at different levels (Manna and Sadhukhan 1991). If the data of embryotoxic effect of the bacterium and that of the fungus *H. oryzae* were compared and subjected to 'Tou' test, at 4th day gestation the value was 4.375 ($P<0.001$), at 12th day it was 24.66 ($P<0.001$) and in the combined data of all intervals it was 53.0 ($P<0.001$) implying that the embryotoxic effect was significantly high for the treatment of *H. oryzae* than that of *X. autotrophicus*. The similar comparison of the embryotoxic data for the treatment of spores of *A. niger* (Table 1) with that of *X. autotrophicus* (Manna and Sadhukhan 1991) yielded the values of 4.14 ($P<0.001$) at 4th day, 20.33 ($P<0.001$) at 12th day of gestation and 75.0 in the combined data of treatment of all intervals. Therefore, like the spores of *H. oryzae*, spores of *A. niger* were also relatively stronger embryotoxic agent when at the same day of gestation treated to normal pregnant mice than that of the bacterial species treated normal pregnant females.

Discussion

Various types of teratogenic effects using mainly mice, rats, rabbits *etc.* as experimental models by different workers for the treatment of ionizing radiations, drugs and chemicals are on record (Woollam 1966, 1967, 1868, 1970). The cuasative factor for the induction of different teratogenic effects by ionizing radiations during development in rats, mice *etc.* (Hicks and D'Amato 1966) was suggested to be the somatic mutation and that dominant skeletal mutation might induce different teratogenic defects as well (Ehling 1970). The treatment of Thalidomite caused numberous cases of human congenital malformation while teratogenic effects were also reported for the use of the insulin, hormones, Azodyes, antibiotics *etc.* (Woollam 1966, 1967, 1968). Among antibiotics induced teratogenic effects recorded in laboratory mammals and some in man in the form of skeletal defects, several other malformations, lethal embryo *etc.* (Fillippi 1967) were Actinomycin D, Adriamycin, Chloramphenicol, Daunomycin, Doxyclicline, Griseofulvin, Hadacins, Mitomycin C, Novobiocin, Oxacillin, Oxytetracycline (Terramycin), Penicillin, Streptomycin, Streptonigrin, Tetracycline *etc.* while no effect was found for the treatment of Kanamycin, Linomycin *etc.* and contradictory results were claimed for the treatment of Daunomycin, Terramycin *etc.*

The studies of teratogenic effects induced by living mutagens except viruses have been limited. The virus associated teratogenic effects reported by different workers were reviewed by Brown (1966). The viral etiology of congenital anomalies were associated with rubella, influenza, cytomegalo, hepatitis, poliomyelitis, measles, mumps, varicella, encephalitis viruses *etc.* The mechanism of teratogenic effects caused by congenital cytomegalo and rubella viruses in infants was suggested to be due to the alteration of foetal anlage by the somatic mutation which caused various malformations (Hanshaw 1970). Among microbes, though viruses have been found to be potential teratogens, our knowledge of similar teratogenic potential of other groups of microbes like bacteria, fungi *etc.* is still very scanty. The culture of the bacterium, *Pseudomonus aeruginosa* treated to pregnant mice on their 1st to 5th day of conception, some amount of lethality and skeletal abnormalities were observed (Chatterjee and Manna 1989) while male parents treated with *P. aeruginosa* and mated to normal virgin females in connection with lethal test (Manna 1985) yielded some embryos with kinking of tail, deformed limbs and some dwarf mutation (Dey 1981, Chatterjee 1986). The embryotoxic effect of another bacterium, *X. autotrophicus* were also observed in mice when normal pregnant females were injected with the bacterial culture at their 2nd, 4th, 6th and 12th day of gestation. Therefore, the embryotoxic potential of two fungal species *H. oryzae* and *A. niger* presented in this paper like that of the bacterium (Manna and Sadhukhan 1991) by us and viruses by others (Brown 1966) would lead towards the generalization that microbes are not only the mutagens shown by us and reviewed from time to time by Manna (1973, 1980, 1986, 1992 a,b) but they are

also potential teratogens. However, the mechanism of embryotoxic effect leading to death of the embryos remained a speculative one. It seemed that some toxic substance produced by the spores of *H. oryzae* and *A. niger* could be the lethal factor but our knowledge about the toxins produced by these fungi is incomplete. They possibly crossed the placental barrier being carried with maternal blood and not by the direct contact to the developing embryos.

Summary

Spores of the fungi, *Helminthosporium orzyae* and *Aspergillus njger* were isolated from the PDA culture medium in sterile distilled water separately and each sample containing 8×10^6 spores per ml of distilled water was intraperitoneally injected @ 1 ml per 100 g body weight to 5 individual normal pregnant mice separately in each set at their 4th, 8th and 12th day of gestation against parallelly injected pregnant mice at the same dose and time with sterile solid slant of PDA medium washed distilled water as controls. The embryotoxic effects leading to lethality of developing embryos were assessed by exposing the uteri of the individual treated and control sets of females at their 15th day of gestation. The data revealed significantly high frequency of lethality to developing embryos in each set of pregnant mice treated separately at their 4th, 8th and 12th day of gestation and in the combined data with spores of *H. oryzae* and *A. njger* than that of control indicating the embryotoxic potential of spores of each species of fungi. The embryotoxic potential of two species of fungi did not show any significant difference between themselves.

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