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Effect of Benzamide on Mitosis & Chromosomes in Mammalian Cells *in vitro*

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In vitro effects of benzamide were studied using primary monolayer cultures from kidney of mongoose. Continuous benzamide treatment affected the mitotic index and chromosomes producing chromosomal stickiness, spindle abnormalities, chromatin bridges, lagging chromosomes and micronuclei. In recovery cultures after 2 hr of benzamide treatment, the chromosomes showed high condensation, chromatid breaks and gaps at short intervals up to 6 hr, however, these effects are not seen in further recovering samples.

BENZAMIDE, a derivative of benzoic acid, has diverse effects on cellular processes in different cell types. In Dipteran polytene cells, benzamide (BM) is known to act as an inhibitor of chromosomal RNA synthesis[1,2]. In plasmodia of *Physarum*, BM has been reported to affect the contractile fibril[3] and amides like formamide have been seen to disrupt the isolated mitotic spindles of sea-urchin eggs[4]. Thus it was of interest to examine the effects of BM on chromosomes and mitosis in mammalian cells. In the present study, kidney cells of mongoose, *Herpestes auropunctatus* Hodgson, have been used to examine the *in vitro* effects of BM.

Materials and Methods

Primary monolayer cultures from kidneys of adult mongoose, grown in TC 199 medium supplemented with 20% bovine serum, were trypsinized and sub-cultured on coverglasses in Leighton tubes. Each sub-culture was initiated with 1 ml of growth medium containing about 1.5×10^6 cells. The following two sets of BM treatments have been done.

Continuous treatment with BM – After 24 hr of medium renewal, 1 mg of benzamide (BDH, Poole) was introduced in each culture containing 1 ml of conditioned medium. After addition of BM, the *p*H of culture medium was readjusted to 7. The experimental and parallel control samples were fixed directly (without colchicine and hypotonic pretreatments) in fresh aceto-methanol (1 : 3) at intervals at 4 hr up to 20 hr and at 30 after addition of BM.

Recovery experiments – After 24 hr of medium renewal, the conditioned medium was removed and saved, and the cells were treated with 0.1% BM in fresh growth medium for 2 hr after the treatment, the cultures were thoroughly washed with Hanks balanced salt solution and the conditioned medium was replaced. Control cultures were left in the conditioned medium. The first control and treated samples were collected 2 hr after BM treatment and subsequently samples were collected at intervals of 4 hr. In each case, colchicine (0.5 µg/ml) was introduced in the cultures 2 hr prior to harvesting. After hypotonic treatment, the coverslips with attached cells were fixed in acetomethanol (1 : 3) for 1 hr and air-dried at room temperature. All preparations were stained with Giemsa (2.5%) at *p*H 7 for 15 to 20 min.

Results

Effect of BM on mitotic index – The effect of different durations of BM treatments on the mitotic index (% metaphase + anaphase among all cells) in mongoose kidney cultures is shown in Fig. 1. From each sample, 1000 cells were randomly scored. In different control samples, the mitotic index ranged between 3.3 to 3.8%. The mitotic index in BM treated cells remained relatively unaffected up to 4 hr however, at 8 and 12 hr, a sharp fall in mitotic index was noticed. The frequency of dividing cells showed a slight rise in samples at 16 and 20 hr but again dropped to a very low value at 30 hr. Analysis of the relative frequencies of metaphase and anaphase figures in BM treated cultures shows that in later samples, the anaphases are less frequent (Table 1).

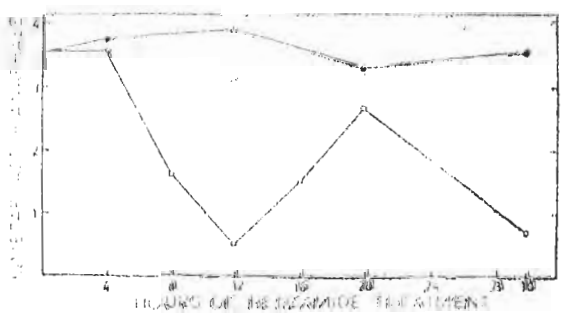

Fig. 1 – Effect of continuous benzamide treatment on mitotic index in kidney cultures of mongoose (○) control; (●) treated)

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TABLE 1 — RELATIVE FREQUENCY OF METAPHASES AND ANAPHASES IN MONGOOSE KIDNEY CULTURES AFTER CONTINUOUS TREATMENT WITH 0.1% BENZAMIDE

Treatment	%Metaphases	%Anaphases*
Control 4 hr	52.0	48.0
BM 4 hr	51.2	48.8
BM 8 hr	55.3	44.7
BM 16 hr	68.6	31.4
BM 20 hr	77.3	22.7
BM 30 hr	87.5	12.5
BM 40 hr	80.8	19.2
Control 40 hr	44	56.0

*Percentage is based on 500 dividing cells from each sample

Effect of BM on chromosomes — Continuous treatment with BM resulted in chromosomal stickiness and other spindle abnormalities in most of the division figures examined in various samples (Fig. 2). Metaphases and anaphases with multipolar spindles were seen frequently (Fig. 2a). Many anaphases stages also showed chromatin bridges (Fig. 2b) and lagging chromosomes. In later samples, the chromatin becomes less clear and seems to be clumped in irregular masses (Fig. 2c). Some metaphases and anaphases in samples up to 16 hr BM treatment revealed presence of nucleolus like material (Fig. 2c). Micronuclei were also occasionally seen in samples collected after 12 hr BM treatment.

Recovery from BM treatment — In general, the mitotic index is not much altered during the recovery periods after BM treatment of 2 hr. The metaphase chromosomes in the 2 hr recovery sample are highly condensed (Fig. 3a) while those in 6 hr and later recovery samples show normal condensation (Fig. 3b-c). The recovery samples at 2 and 6 hr show chromatid and isochromatid gaps in some metaphases (Fig. 3b). However, these gaps are not seen in later recovery periods. The centromeric regions of metaphase chromosomes seem to be the regions most affected by short BM treatment. The centromeric regions of different chromosomes show stickiness and are often drawn together in groups forming rosettes (Fig. 3c). Also, in some of the metaphase plates, the centromeric regions of all chromosomes are seen to be attenuated (Fig. 3b) and often the sister centromeres show precocious separation. These effects on centromeres are commonly seen in 6 hr recovery sample but later metaphases appear nearly normal. Thus it appears that complete recovery from effects of short BM treatment is achieved after culturing the cells in BM-free medium for periods longer than 6 hr.

Discussion

The major effects of a prolonged BM treatment on mitotic cells of mongoose appear to be on chromosome condensation and spindle organisation. Chromosomal aberrations were conspicuously rare in BM treated cells. McGill *et al.*[5], have suggested

Fig. 2 — Effects of continuous benzamide treatment on mitosis and chromosomes in kidney cultures. (a) a tripolar anaphase; (b) anaphase showing chromatid bridges; and (c) metaphase with irregularly clumped chromosomes and persistant nucleolar material (arrows)

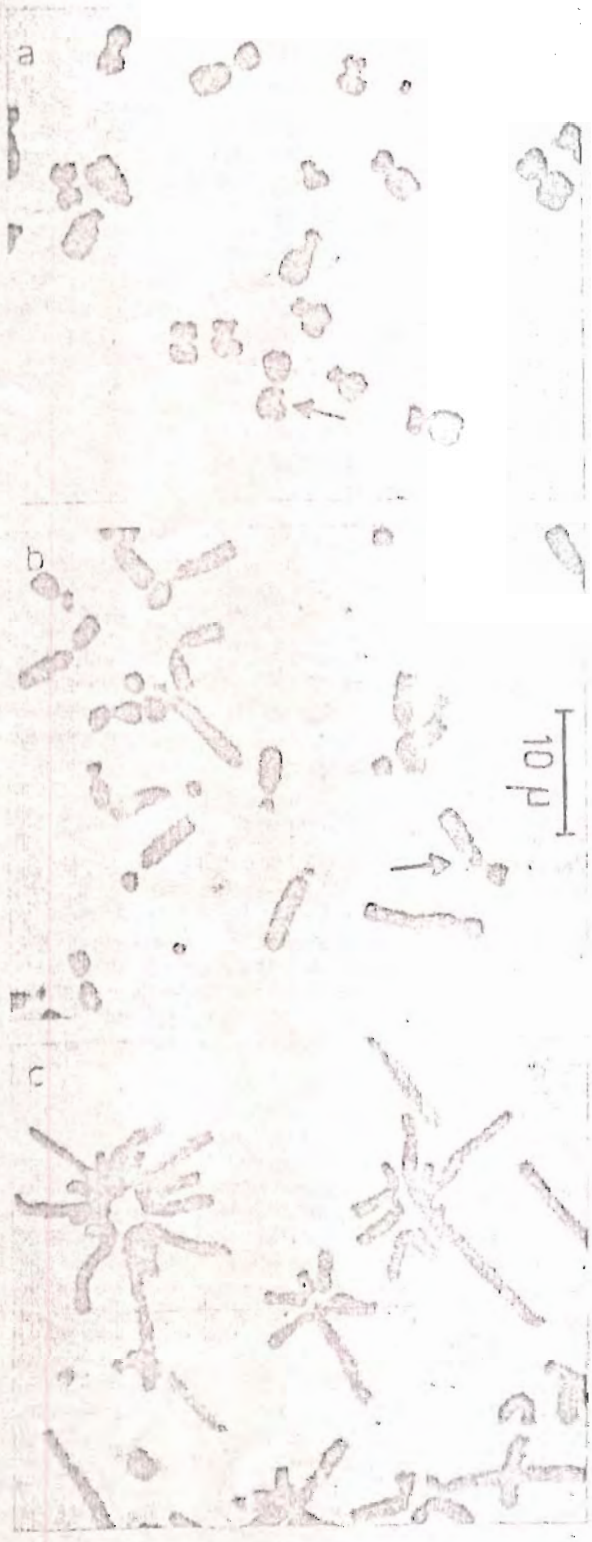

Fig. 3 — Partial metaphases from 2 and 6 hr recovery samples after 2 hr benzamide treatment, (a) highly condensed chromosomes seen in 2 hr recovery sample; (b) stretching of centromeric regions of all chromosomes and chromatid gap in one chromosome (arrow) in 6 hr recovery sample; and (c) stickiness of centromeric regions resulting in rosettes of groups of chromosomes in 6 hr recovery sample

that the stickiness of chromosomes and presence of anaphase bridges after treatment with certain clastogens may be due to abnormal condensation of chromatin so that fibrils of different chromosomes may get trapped and entangled with each other. Presumably, the BM treatment also disturbs the normal chromosome condensation in a similar manner which results in chromosomes stickiness and anaphase bridges as noted by us in continuously treated cultures. McGill *et al*[5] and Pathak *et al*.[6], discussed the role of chromosome stickiness in causing chromatic breaks at later stages, presumably in 2nd division cycle. In the present material continuously treated cells did not display many breaks, although chromosome stickiness was very commonly seen. It appears that after a prolonged BM treatment, most of the mitotic cells are so badly damaged that they do not enter 2nd mitosis and thus any potential aberration is not seen. The gradual decline in the relative abundance of anaphases and also the preponderance of abnormal division figures in later BM treated samples are indicative of mitotoxicity of benzamide. However, the breaks resulting from anaphase separation of sticky chromosomes[5] in BM treated cells are presumably manifest in micronuclei seen in some interphase cells in samples collected after 12 hr BM treatment.

Effect of BM on chromosome condensation is also evident in the recovery experiment. In the 2 hr recovery sample, colchicine was added to the cultures immediately after removal of BM so that different metaphases would have recovered for 0 to 2 hr after the BM treatment. In this sample, metaphase chromosomes were seen to be highly contracted whereas in 6 hr and later samples, the overall condensation of metaphase chromosomes was as in normal colchicine treated cells. It may, therefore, be suggested that the G_2 phase is the sensitive period, during which presence of BM may cause excessive chromosome condensation. Presence of occasional gaps in 2 and 6 hr recovery, but not in later samples, also indicates the sensitivity of late S and G_2 periods to BM.

The stickiness and elongation of centromeric regions in mongoose chromosomes seen during recovery after 2 hr BM treatment, is very interesting. Again since these effects on centromeres are not seen in later recovery periods, it appears that the action of BM on centromeric regions occurs specifically either during late S (the time when centromeric regions are replicating[7]) or in G_2. The underlying causes for these effects on centromeres are not known, but it is interesting to note that a similar stickiness of centromeres of different chromosomes is not induced by BM in *Rattus rattus* although centromeric elongation is observed[2]. Thus the effects seen in present study may be due to some specific feature of centromeric organization of mongoose chromosomes.

In general, the recovery experiments thus suggest that the short term effects of BM on mongoose chromosomes may be restricted to late S and G_2 periods since the samples collected after 6 hr recovery show near normal mitosis. Presence of multipolar spindles and disorganized metaphase - and a decrease in anaphase frequency in cells continuously exposed to BM for long periods are indicative of effects of

BM on the mitotic apparatus. While examining the effects of ethidium bromide, McGill *et al.*[5] observed various spindle and centriolar abnormalities and suggested that the abnormal spindle organization in treated cells may be due to abnormal behaviour of centrioles (also see ref. 8). Presence of similar spindle abnormalities in the BM treated mongoose cells thus suggest that like ethidium bromide[5] BM may also alter centriolar organization.

The precise mode of action of BM on mongoose cells is not known. In dipteran polytene cells, BM strongly inhibits chromosomal RNA synthesis in preference to nucleolar synthesis[1,2]. However, in mammalian cells, a similar action of BM may not be present, since it has been observed in our laboratory that even a prolonged incubation of HeLa cells in presence of BM does not have any significant effects on RNA synthesis[9]. Therefore, it appears that the effects of BM on mongoose mitotic cells may not be due to any major disturbances in cellular RNA synthesis. It may be noted in this context that actinomycin D, a strong inhibitor of RNA synthesis induces cross-bands in treated metaphase chromosomes[6], while no cross-bands were seen in BM treated mongoose chromosomes.

Korohoda and Wohlfarth-Bottermann[3] have observed that in plasmodia of *Physarum*, BM can induce local relaxation by degradation of actomyosin fibrils. Nevo *et al.*[4], have reported disruption of isolated mitotic spindle of sea-urchin eggs by treatment with several amides. In view of these reports and our present observations on the various mitotic abnormalities induced by BM in mongoose cells, it would be interesting to examine the effects of BM on microtubules and other cellular contractile elements in mammalian cells.

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