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"The Effects of Hypoxic Radiosensitizing Drugs on *In Vitro* Tissues of the Central Nervous System"

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SUMMARY

The aerobic cytotoxicity of metronidazole and misonidazole has been studied using primary explant tissue from the central nervous system (CNS) of the rat. Both chronic and acute exposure paradigms were used in conjunction with endpoint measurements of culture area growth and single unit electrophysiology to generate a qualitative dose response function for these drugs on *in vitro* CNS tissue.

Our results have demonstrated a clear aerobic cytotoxicity of these drugs on this *in vitro* CNS tissue. The neurotoxicity was both dose- and culture age-dependent and was manifest as a reduced growth in culture area. Misonidazole was toxic at concentrations of 5 mM or greater regardless of the culture's age, while metronidazole was only harmful at a 10 mM concentration in younger cultures. The age-dependency is probably due to the altered metabolic state of this CNS tissue during this time period.

Neuronal action potential ("spike") electrogenesis was monitored online during acute exposures to misonidazole under aerobic conditions. Complete and irreversible loss of spike electrogenesis was seen with 1 mM concentrations while significant, but reversible, effects were obtained with 100 uM concentrations. These preliminary data suggest a potential aerobic cytotoxicity of misonidazole in CNS tissue at clinically relevant doses.

Introduction

The role of ionizing radiation therapy has been firmly established as an alternative to invasive surgery for a wide variety of malignant tumors, especially in the brain. However, the presence of radioresistant, hypoxic cells in tumors has limited the overall radiotherapeutic gain. The use of high LET, heavy ion radiotherapy is, of course, currently being developed at LBL as an important approach to deal with hypoxic tumor cell populations (Blakely et al., 1980; Castro, 1980).

Hyperbaric oxygen and hypoxic cell radiosensitizing agents are two adjunct therapy protocols which have also been utilized in an effort to overcome the relative radioresistance of hypoxic tumor cells (Churchill-Davidson, 1967; Wasserman et al., 1979). The danger of oxygen toxicity and the technical problems in using the hyperbaric chambers have limited the overall usefulness of the former approach. The latter approach is currently dose-limited due to deleterious side-effects of the radiosensitizing agents, and mechanistic explanations of their radiosensitization are still being investigated.

The nitroimidazoles, metronidazole and misonidazole, are hypoxic cell radiosensitizers which are currently being tested in Phase II and III clinical radiotherapy trials (Wassermann et al., 1981; Phillips et al., 1982). While preliminary results indicate improved survival statistics for radioresistant tumors (Wasserman et al., 1979), various complications arising from the clinical use of these drugs have been of considerable concern. In addition to the acute gastrointestinal effects, neurological toxicities have been documented. These include peripheral neuropathy and its associated sensory paraesthesia (Urtasun et al., 1977; Dische et al., 1977), convulsions (Dische et al., 1979), ataxia (Sealy, 1978), muscle atonia (Wasserman et al., 1979), and ototoxicity (Wasserman et al., 1979).

A substantial body of evidence exists relative to the effects of these drugs on the peripheral nervous system (PNS) (Urtasun et al., 1978; Griffin et al., 1979). While the initial report of a decrease in peripheral nerve conduction velocity (Hirst et al., 1978) has not been substantiated (Von Burg et al., 1979), evidence does exist that strongly implicates damage to the sensory nerve terminals on intrafusal fibers, as well as the distal axonal segments of the sciatic and posterior tibial nerves of the PNS (Griffin et al., 1979; Rose et al., 1982).

One of the issues still outstanding is the extent to which the central nervous system (CNS) is adversely affected by the clinical doses employed with these hypoxic cell radiosensitizing agents. Ototoxicity, lethargy, encephalopathy, confusion, coma, and convulsions are deleterious responses associated with the CNS (Dische et al., 1976; Wasserman et al., 1979). Furthermore, animal studies carried out with dose levels comparable to the clinical setting have also demonstrated a significant deficit in locomotion and balance, which suggested a CNS deficit, in some cases without gross morphological damage (Conroy et al., 1979). Other detailed morphological work has, however, demonstrated significant CNS neurotoxicity in rats given 260 and 360 mg/kg misonidazole injections (5/week; ip) for 3 weeks to 5 months (Griffin et al., 1979). The neurological damage was extremely localized and was found primarily in the lateral vestibular nucleus and superior olive, as well as in the cochlear nucleus and cerebellar roof nuclei. Behavioral deficits and corresponding morphological alterations in the cerebellar Purkinje cells of dogs has also been reported (Schärer, 1972). A recent study (Powers et al., 1982) reported that significant alterations occur in rodent brain stem auditory evoked potentials prior to the cumulative dose level of approximately 5.8 gm/kg that resulted in clinical symptoms of CNS neurotoxicity. Relatively few clinical studies

with hypoxic cell radiosensitizers have systematically studied the CNS dysfunction component of neurotoxicity.

This preliminary report addresses the question of whether metronidazole or misonidazole have any deleterious effect(s) on normal CNS tissue growth, development, and function (action potential electrogenesis). To test the dose-dependent effects of these drugs on CNS tissues, we have used explant cultures of neonatal rat cerebellum as an in vitro bioassay system. This neurotoxicological model system has already been used to determine the direct effects of hypoxia and carbon monoxide insults on CNS tissue (Raybourn et al., 1978; manuscript in preparation). This in vitro system has been shown to be a good model for the cyto- and organotypic characteristics of the in vivo cerebellum at the morphological, biochemical, and electrophysiological levels (Lehrer et al., 1970; Woodward et al., 1969; Crain, 1976). It is an excellent system for testing both acute and chronic CNS effects of radiosensitizing agents. Moreover, spontaneous action potentials are seen in extracellular microelectrode recordings from single Purkinje cells in these monolayer cultures, and evidence for synaptic interactions has been demonstrated (Schlapfer et al., 1972). It is this synaptically modulated, spontaneous action potential activity (spike electrogenesis) that has provided us with a direct assessment of neuronal function in this in vitro system.

The cerebellum has been implicated as a sensitive target for the effects of radiosensitizers. The reported CNS effects of hypoxic radiosensitizers (Griffin et al., 1979; Conroy et al., 1979) have been on neuronal structures (lateral vestibular nucleus, superior olive, deep cerebellar nuclei, etc.) which have an intimate structural and functional relationship with cerebellar tissue. Moreover, cerebellar Purkinje cells were reported to be selectively damaged by nitroimidazoles (Schärer, 1972). Several of the reported clinical

manifestations of hypoxic cell radiosensitizer neurotoxicity (ataxia, locomotion deficits) are also obtained after selective cerebellar lesions (Llinas et al., 1975; Llinas and Walton, 1979). Our report examines the cytotoxic effects of metronidazole and misonidazole on the growth and electrophysiological competence of neonatal rat primary cerebellar explants *in vitro.*

Materials and Methods

Culturing Technique

The primary cerebellar tissue explants (1 mm^3) were obtained from 3-4 day old Sprague-Dawley rat pups. The explant was attached to a glass coverslip on a plasma clot, supplemented with medium and incubated without antibotics at 37°C in CO_2 gas-diffusible test tubes (Falcon #3003) using the flying coverslip-roller drum incubation method (Murray, 1965). The incubation media consisted of 50% minimal essential medium, 25% fetal calf serum, and 25% Earle's BSS. This was supplemented with glucose at 6 mg/ml. The medium was replaced twice weekly and was maintained at pH of 6.9-7.2 by a constant infusion of 5% CO_2 gas into the incubator. After 10-14 DIV (days *in vitro*), the explant cultures spread out and thinned into a monolayer. At this time individual Purkinje cells could be visualized with Nikon phase contrast optics (Schlapfer et al., 1972; Raybourn et al., 1978).

Drugs

The hypoxic radiosensitizing drugs were provided by Dr. Harry Wood of the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment, National Cancer Institute. Metronidazole, 2-methyl-5-nitroimidazole, $C_6H_9N_3O_3$, (m.w. = 171.15), and misonidazole (RO-07-0582), 1-(nitro-1-imidazole)-3-methyoxy-2-propanol, $C_7H_{11}O_4N_3$ (m.w. = 201.18) were supplied in a powder form and were

added directly to the culture medium. They were dissolved by warming at 37°C for three to four hours and filter sterilized under vacuum with 0.45 μm millipore filters. Because these compounds are light-sensitive, all handling was done with minimal exposure to light, and they were stored in shielded containers at 4°C in between culture feedings.

Drug Incubation Protocols

Chronic - At a specific time during the culture's growth period, either (1) early (3-4 DIV), or (2) late (10-11 DIV), the normal incubation medium was exchanged for one containing 1.0, 5.0, or 10.0 mM of either misonidazole or metronidazole added to the medium and bicarbonate-buffered to a pH of 7.0. All subsequent medium replacements contained the appropriate drug. Control cultures were fed with medium without drugs according to the same schedule used for the experimental groups.

Pulses - In these experiments, the normal culture medium was exchanged for one containing either 1 or 10mM of metronidazole or misonidazole for either 60 minutes, 24 hours, or 48 hours. After the drug-medium was removed, the cultures were gently rinsed five times with BSS and placed in a new tissue culture tube containing normal medium. Subsequent medium exchanges were with normal medium. The control cultures were similarly fed, except with medium containing no drugs.

Acute - This experimental regimen was only carried out after the culture was removed from its incubation tube and placed in the liquid perfusion system of the electrophysiological recording chamber (see below). The culture was continuously bathed (0.5-1.5 ml/min.) with an air-bubbled, glucose-fortified Hank's BSS solution. At some time during the electrophysiological recording session, the perfusion delivery system was switched from the normal BSS reservior to one contianing air-bubbled BSS with either 0.1 mM or 1.0 mM misonidazole.

This continued for a variable length of time before the perfusion line was switched back to the normal BSS reservoir for the return-to-control measurements.

Area Growth Measurements

To monitor the rate at which the explants spread into a monolayer on the coverslips, the cultures were measured two to three times per week and the explant area calculated. Measurements were obtained by placing each tissue culture tube in a specially designed holder which provided a perpendicular view of the glass coverslip containing the explant culture. Each culture was viewed with a stereo dissecting scope (120x magnification) and the diameter measured with an ocular reticule (0.1 mm resolution). Although most explants grew in an approximately radial direction, those that were asymmetrical were measured in two orthogonal dimensions and the resultant area calculated. Any holes or circumferential irregularities were likewise measured and their area subtracted from the overall explant area to provide the net culture area.

Electrophysiology

Extracellular microelectrode recordings were obtained from electrically isolated single Purkinje cells in cultures from 14-28 DIV. All cultures were chronically incubated under normal conditions and with normal medium. After a brief morphological examination under Nikon phase contrast optics, the coverslip containing the cerebellar explant was placed in a temperature-controlled ($37^{o}C$) liquid perfusate-bathed recording chamber. This chamber was mounted on the stage of an inverted phase contrast microscope. The pH, temperature, and pO_2 of the perfusate were monitored in the recording chamber. Glucose-fortified Hank's BSS was used as the normal perfusate medium. A gravity-drain, liquid perfusion system provided constant flow rates (0.5-1.5 ml/min.), and one of the three separate inlet syringe reservoirs could be selected to

provide different BSS solutions (e.g., normal) air-bubbled BSS, air-bubbled BSS with radiosensitizing drug, or N_2-bubbled BSS). Equilibrium measurements of the oxygen tension of the 95% N_2 - 5% CO_2 bubbled BSS in the recording chamber indicated a dissolved pO_2 of 1.3-1.5 ppm. This represents a physiological, but not radiobiological, level of hypoxia (Koch and Biaglow, 1978). Depending on the flow-rate, the initial bolus of the new BSS reached the recording chamber in 0.5-1.5 minutes.

The electrophysiological recordings were obtained with 2-5 μm tip diameter glass micropipettes, filled with 2M NaCl, and loaded in a FET pre-amplifier head-stage, all of which was mounted on a motor-driven, three-dimensional microdrive (1 μm resolution of movement). The A-C coupled pre-amplifier head was connected to the main differential amplifier (Model DAM-5A; WP-Instruments) which was operated with a 300-5,000 Hz band-pass frequency response. Its output was fed to a custom-designed, voltage window discriminator whose digital output went to an audio amplifier, a magnetic tape recorder, a specially modified pulse height analyzer, and an integrating ratemeter with a 5-second integration time. The multiplexed output of the window discriminator was fed to an oscilloscope for online monitoring, and the output of the ratemeter was transferred to the strip chart recorder. The details of this electrophysiological recording system were given in Raybourn et al., (1978).

The acute experiment began when a spontaneously active Purkinje cell was electrophysiologically isolated with a reasonable activity rate (5-20 spikes per second; CPS), and a minimal 2:1 signal:noise action potential criterion was met. Control data samples were then collected of the cell's instantaneous firing frequency over a 5-10 minute period. After control data were obtained with no BSS perfusate flow and, then after the BSS flow was begun, the experimental manipulations were initiated. In all cases a minimum data collection

period of five minutes per condition was employed in order to obtain a statistically reliable sample.

Results

Culture Area Growth

Chronic Studies - A total of six separate experiments were carried out using chronic drug incubation protocols. Three of the studies were conducted with the chronic drug treatment starting at 3-4 DIV, while in the other three studies the chronic drug incubations were begun at 10-11 DIV. In all test conditions there were six cultures per dose level for each drug type. An equal or greater number of control cultures was monitored in each study.

When either metronidazole or misonidazole was used at a 10 mM concentration with 3 DIV cultures, a clear reduction in final net culture area was observed relative to the control group, by the end of the observation period. This is demonstrated in Figure 1 for two separate experiments. The stippled area represents the standard deviation about the mean of the control cultures. Although the variance obtained with these structural endpoint measurements is fairly large, both radiosensitizing drugs had an obvious adverse effect on the growth of aerobic CNS tissues at a 10 mM concentration level. However, when metronidazole and misonidazole were tested at a 5 mM concentration from 4 DIV onwards, only misonidazole produced any significant reduction in culture area growth relative to control cultures. This is shown in Figure 2A. Other experiments (data not shown) demonstrated that no deleterious effects of either drug were obtained at 1 mM concentrations with these 3-4 DIV cultures.

A different picture emerged when older (10-11 DIV) cultures were chronically incubated with metronidazole and misonidazole at 10 mM concentrations.

In Figure 3 it can be seen that only the misonidazole exposure resulted in an appreciable decrement in culture area growth in two separate experiments. The means and standard deviation for metronidazole fall well within the range of the control cultures. A separate experiment with 1 mM concentrations of both drugs on 10 DIV cultures did not result in any reduction of culture growth relative to controls for either drug (Figure 2B).

Pulsed Studies - The results of a sixty minute exposure of 1 DIV cultures to 1.0 mM or 10.0 mM of misonidazole are seen in Figure 4. An inhibition of culture area growth is seen by 6 DIV at the 10 mM dose level in Figure 4A. The 1 mM misonidazole exposures had no significant effect relative to controls. A replication of this experiment yielded the same results except for a more marked inhibition with 10 mM misonidazole by 7 DIV (Figure 4B).

Two experiments were also carried out with 24 and 48 hour pulse incubations of 10 DIV cultures with 10 mM misonidazole and 10 mM metronidazole (Table I). In the 24 hour exposure study the 10 mM misonidazole group showed a final net culture area deficit relative to both the controls and the 10 mM metronidazole group. The latter group did not differ from the controls. In the 48 hour pulse study, the same results were seen as with the 24 hour pulse, except that the 10 mM misonidazole group exhibited a larger and more rapid overall decrement relative to both the control and 10 mM metronidazole groups. The latter group, again, did not differ from the controls.

Electrophysiology

Even though misonidazole (at a 5 mM or greater) proved to be the more potent drug for the older aerobic CNS tissues ($\geq$ 10 DIV) on the basis of the culture area growth data presented above, the preliminary studies reported here on the electrophysiological responses of *in vitro* Purkinje cells were begun at the 1 mM dose level because we anticipated a greater sensitivity

with this functional bioassay. Since the electrophysiological recordings could not be carried out with cultures younger than 12-14 DIV (because of the time required for the monolayer culture to develop), metronidazole was not tested because of its lack of effect on 10 DIV cultures in the area growth measurements. Although the preliminary electrophysiological studies reported here represent only a limited number of experiments (and therefore any definite conclusions await the generation of a larger data base), the use of a return-to-control protocol for each Purkinje cell studied constitutes a highly reliable analysis of the effects of these radiosensitizing agents on neuronal function.

In Figure 5 the instantaneous firing frequency of action potentials from a Purkinje cell in a 19 DIV culture is presented as a function of control and 1 mM misonidazole BSS exposures. The chart recorder record begins with the spontaneous firing frequency seen under a static (no flow) liquid perfusate condition. Three and one-half minutes later, the control (air-equilibrated) BSS flow was begun. There was a transient, hydrodynamically caused decrease in firing frequency followed by a return to a stable firing rate. Twelve minutes into the experiment a 1 mM misonidazole-containing BSS flow (air-bubbled) was exchanged for the normal BSS flow. After a short delay (partially due to the flow kinetics of the liquid perfusate system), a large and somewhat irregular decline in the cell firing rate was seen. By ten minutes the instantaneous firing frequency had dropped from around 10 CPS to 3 CPS. At that point the control BSS flow was reinstituted. Although the cell was monitored for an additional fifteen minutes, there was no return-to-control firing activity.

These data can be contrasted with the usual 90-240 minute duration of online recordings that are obtained under control conditions. This dosage (1 mM) of misonidazole was tested on several other Purkinje cells (N=3) electrophysiologically isolated in different cultures; however, either the appropriate

control criteria were not met or the cell was lost before the entire experimental sequence could be achieved. In none of these partial experiments, however, was any recovery of Purkinje cell activity ever seen after exposure to 1 mM misonidazole.

In order to begin the preliminary determination of a functional dose response relationship for the effects of misonidazole on aerobic CNS neurones, we tested the effects of an acute 0.1 mM (100 μM) misonidazole exposure on the spontaneous activity of in vitro cerebellar Purkinje cells. Again, as in the 1 mM misonidazole data (Figure 5, the data shown here in Figure 6 for a 15 DIV culture are indicative of a decided effect of this radiosensitizing agent on aerobic CNS neuronal function. However, unlike the results obtained at 1 mM, an acute 0.1 mM (100 μM) misonidazole exposure was reversible. In Figure 6 the same hydrodynamically derived perturbation was seen in the instantaneous firing frequency following the onset of the liquid perfusate flow. After stabilization of the firing rate, a 95% N_2 - 5% CO_2 gas-bubbled BSS was introduced into the recording chamber (which resulted in a dissolved pO_2 of 1.3-1.5 ppm). After a one-to-two minute delay, a clear decrease in firing rate was seen (6.5 to 2.5 CPS). After we returned to the control BSS (air-bubbled) perfusate, the cell recovered its normal firing rate, perhaps with a small overshoot. Thirteen minutes into the recording session, a 100 μM misonidazole-BSS flow was turned on. A drop in firing rate, very similar to that seen with the hypoxic BSS insult, soon occurred. After two minutes the control BSS flow was reinstituted. The return-to-control kinetics from this insult are comparable to those following the hypoxic insult. A second exposure to the same 100 μM misonidazole followed the return-to-control firing levels. Although the kinetics fo the decline in firing rate were slightly slower than those seen in the first acute exposure, the absolute level obtained was the same. After eight minutes the control BSS flow was reinstated. Unlike

the initial exposure, the return-to-control firing levels took almost eight minutes in this second exposure, reflecting the longer drug exposure time.

Discussion

Culture Area Growth

Experiments conducted with varying durations of exposure to these radiosensitizing drugs, under aerobic conditions, have shown that with continuous chronic incubation protocols, both metronidazole and misonidazole (Figure 1) have decidely negative effects at 10 mM concentrations with explant cultures challenged at an early age (3-4 DIV). However, at 5 mM doses (Figure 2A) only misonidazole had any deleterious effects, suggesting a more toxic effect of misonidazole on aerobic CNS tissue.

The 24 and 48 hour pulsed incubation studies with 10 DIV cultures (Table I), which approximate the body retention half-lives of these agents in humans (Dische et al., 1979), demonstrated that long-term chronic incubations are not necessary to elicite CNS neurotoxicity. Moreover, when 1 DIV cultures were challenged with 10 mM misonidazole, even a 60 minute pulse duration was sufficient to result in significant neurotoxicity (Figure 4). This can be contrasted with the 40-70 hour exposure to 10 mM misonidazole that is required for nonneural V-79 cell toxicity (Stratford, 1979).

Furthermore, the culture area measurements indicate that a significant difference in radiosensitizing drug neurotoxicity is seen with chronic incubations of younger (3-4 DIV) versus older (10-11 DIV) cultures. That is, only misonidazole (at 10 mM) retains its neurotoxicity in the older explant cultures. The importance of this finding lies in the fact that the developmentally controlled metabolic state of this CNS tissue (*in vitro* and *in vivo*) changes dramatically over this time period, suggesting that the nature and level of

bioenergetic activity in neural tissues can influence the aerobic neurotoxicity of these radiosensitizing drugs.

While it is clear that these drugs exhibit oxygen-mimicking effects in hypoxic tissues (Hall and Roiziu-Towle, 1975), and that their one-electron reduction potentials are correlated with their radiosensitizing capacity (Adams et al., 1976), the actual molecular basis for their action on aerobic tissues is still a matter of study. A recent study (Olive, 1982) has suggested that the actual damage mechanism is the same for both aerobic and hypoxic tissues. Their decided electron affinity (Olive, 1979) and capacity to alter mitochondrial oxidative phosphorylation and oxygen consumption (Mustea et al., 1978; Mustea and Bara, 1979; Koch and Biaglow, 1978) suggest that these hypoxic radiosensitizing agents might interfere with cellular mechanisms relating to ATP synthesis. The deleterious role of these agents in cellular bioenergetics (especially in the CNS) has only begun to be studied (Varnes and Biaglow, 1982; Chao et al., 1982; Conroy et al., 1982), but a recent suggestion (Olive, 1979b,c) is that the aerobic effects of these agents are "cytostatic" in nature rather than cytotoxic. That is, there is a slowing down of cell cycle progression (Deys and Stap, 1979; Lindmo et al., 1979), with a resultant disruption of growth-supporting functions.

The differences in metronidazole neurotoxicity seen with 3-4 DIV versus 10-11 DIV cultures are best understood in the context of the developmental changes that occur in cerebellar tissue, especially in the Purkinje cells, during this 2-8 DIV time period (or during the same time *in vivo*). The outward growth of the cultures in these *in vitro* cerebellar explants is primarily due to movement of both glial cells (oligodendrocytes, astrocytes) and mesodermal elements (epithelial cells, fibroblasts, and macrophages), as well as neurite extensions from the existent nerve cells (Purkinje, granule, Golgi, and basket cells). During this time period there is a massive activation of enzyme systems

(hexokinase, succinate and lactate dehydrogenase, and GABA ketogluterate transaminase) involved in glucose-based energy metabolism (Lehrer et al., 1970; Woodward et al., 1969; Simurda and Wilson, 1980). This enhanced glycolytic activity precedes the emergence of the apical dendrite from the Purkinje cell soma at about 10 days postnatum (Addison, 1911). It is also coincident with the extensive granule cell proliferation and migration (Altman, 1972b), as well as the oligodendrocyte proliferation and differentiation prior to the myelination process, which also begins at around 10 days postnatal in vivo (Lunsden, 1958) and in vitro (Mamoon, 1969). The decrease in culture area growth would seem to reflect the aerobic cytostatic effect discussed by Olive (1979a,b,c).

In terms of this developmental picture, the current data suggest that, at 3-4 DIV, both metronidazole and misonidazole have significant aerobic neurotoxic effects on cerebellar tissue development, perhaps by dysfunctional perturbations in cellular glycolytic metabolism. However, by 10-11 DIV the extensive activation of glycolytic-based bioenergetic processes has lessened considerably, and only misonidazole (at 5-10 mM) exposures result in aerobic neurotoxicity. While this greater overall toxicity of misonidazole, as compared to metronidazole conflicts with previous data from both the clinical and experimental literature (Adams et al., 1976; Adams et al., 1979), we believe that our report is the first comparative assessment of their toxicities in the CNS. Furthermore, although the aerobic cellular toxicity exhibited by both radiosensitizing drugs in clearly related to their one-electron reduction potential (Biaglow et al., 1978), our data indicate that the metabolic state of neural tissues is also quite important in determining the resultant neurotoxicity.

It is also important to note that the reductions in net culture area

caused by chronic incubations with these radiosensitizing drugs only occurred at concentrations (5-10 mM) that exceed the usual clinical dose levels ($\leq$ 1 mM). Although not presented in this paper, these findings were subtantiated by light microscopic analysis of the tissue and cellular morphology that was carried out in parallel with the area growth measurements and that showed no deleterious effects at 5 mM or less. Moreover, the single cell electrophysiology data (vide infra) clearly demonstrated significant perturbations of neuronal function (spike electrogenesis) at 100 μM levels of misonidazole. Irreversible loss of function was observed at 1 mM, a dose level that did not cause any negative structural effects as assessed by the area growth measurement technique. Although qualitative aerobic cytoxicity dose-response functions can be obtained, our data strongly suggest that structural endpoints (e.g., tissue area, volume, morphology) lack the sensitivity to critically assess threshold levels of toxicity by radiosensitizing drugs on neural tissue.

Electrophysiology

To our knowledge, the data presented in Figures 5 and 6 represent the first demonstration of the acute effects of a radiosensitizing drug on the functional viability of a CNS neurone. Although the small sample size precludes statistical conclusions, several aspects of these preliminary data warrant special emphasis. First of all, it is clear that a functional bioassay technique, such as the online electrophysiology used here, is very sensitive to the oftsubtle variations in neuronal cell function(s) that may underlie CNS neurotoxicity. Even though a qualitative dose response function can be derived from culture area measurements, the observation that 1 mM misonidazole or 1 mM metronidazole had no aerobic neurotoxicity (from morphological inspection) was obviously limited by the less sensitive nature of that analysis.

A second important conclusion from these data is the direct comparison of hypoxia and 100 uM misonidazole on the same Purkinje cell (Figure 6).

The similarity of neuronal response to these cellular insults suggests that a common process may have been perturbed. The effects of hypoxia on the *in vivo* CNS are reasonably well understood (Schade and McMenemy, 1963), and the *in vitro* response of neuronal tissues and components has also been addressed (Swanson, 1969; Kim, 1975; Albrecht and Smialek, 1975). The specific responses of this *in vitro* cerebellar preparation to hypoxia and carbon monoxide was also characterized (Raybourn et al., 1978; manuscript in preparation). In sum, as the partial pressure of oxygen is lowered, the ability of the terminal cytochrome aa_3 oxidase molecule to participate in electron transfer is lessened, and anaerobic glycolysis is likewise activated (Lowry and Passonneau, 1964; Bachelard et al., 1974; Sussman et al., 1980). In terms of plasma membrane functions, there is a depolarization of the membrane resting potential (Auclair et al., 1976) and a high-energy phosphate depletion (Härkönen et al., 1969), both leading to a cessation of spike electrogenesis. *In vivo* spectrofluorometric data (Chance et al., 1962) indicate that NAD becomes increasingly reduced under hypoxic conditions. The role of NADH in the control of the overall functioning of the electron transport chain is well-known (Chance and Hess, 1959).

The role of radiosensitizing drugs on cellular metabolism has been addressed with various tumor and nonneural mammalian cell preparations (Mustea et al., 1978; Koch and Biaglow, 1978; Biaglow et al., 1978; Biaglow, 1980), and it has been shown that these drugs can alter cellular respiration and mitochrondrial oxidative phosphorlyation. The drug's one-electron reduction potential was not a very good predictor of these obtained effects (Biaglow et al., 1978). All of these studies have suggested that the locus of the effects on cellular metabolism of these radiosensitizing drugs is at, or prior to, the level of the flavoproteins (NAD/NADH) in the respiratory chain. Other data have addressed

the importance of the metabolic state of aerobic tissues in their resultant cytotoxicity to radiosensitizing drugs (Mustea and Bara, 1979; Varnes and Biaglow, 1982; Chao et al., 1982). In brief, it appears that these agents result in a considerable inhibition of cellular glycolysis, as well as in significant pertubrations of oxygen consumption. Since normal CNS tissues function in a relatively hypoxic (reduced) state to begin with (Hempel, 1979), the potential interaction of the nitroimdazoles and these neural tissues may apply under both the aerobic and hypoxic conditions.

Our results, both at a structural and functional level of analysis with these in vitro CNS tissues, indicate that: (1) a definitive aerobic CNS neurotoxicity of metronidazole and misonidazole does exist; and (2) that the mechanism(s) of this deleterious effect is probably related to a disruption of the cellular processes providing bioenergetic functions to CNS neurons. This latter conclusion is derived both from the rather rapid kinetics of spike electrogenesis failure seen in our acute experiments relative to the time constants involved in perturbations of DNA synthesis, and from the similarity of the electrophysiological responses to these hypoxic radiosensitizing drugs and acute hypoxia under aerobic conditions.

The relatively high rate of cellular respiration in CNS neurones (McIlwain, 1966), in addition to the relatively hypoxic microenvironment in which they normally function (Hempel, 1979) suggests that normal CNS tissues may possess a significant susceptability to the deleterious side-effects of the nitroimidazole radiosensitizers. The lack of a significantly analytical clinical data base on radiosensitizer-induced CNS dysfunction, as compared to the demonstrated PNS effects, remains the major limitation in the assessment of the relative importance of these present findings.

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Figure Legends

Figure 1 - Effects of chronic incubation of 10mM metronidazole or 10mM misonidazole under aerobic conditions on the *in vitro* growth of cerebellar explant tissue challenged from 3 days *in vitro* (DIV) onwards. Both drugs eventually depress the final culture area below that of control cultures. Figures 1A and 1B are two separate experiments. The stippled area in this and all subsequent figures represents the standard deviation about the control group means. Error bars represent the standard deviation about the experimental group means. (XBL 827-4002B)

Figure 2 - A: Effects of chronic incubation with 5mM metronidazole or 5mM misonidazole starting at 4 DIV. Only misonidazole had any significant effect on culture area growth. B: Effects of chronic incubation with 1mM metronidazole or 1mM misonidazole begun at 10 DIV. Neither drug had any significant effect versus the control group. (XBL 828-4041)

Figure 3 - Two separate experiments (A,B) with older (10-11 DIV) cerebellar cultures chronically incubated with 10mM metronidazole or 10mM misonidazole. Unlike results obtained with younger cultures (3-4 DIV), only misonidazole continued to suppress explant area growth. (XBL 827-4001A)

Figure 4 - Effects of pulsed incubation, under aerobic conditions, with either 1mM or 10mM of misonidazole on 1 DIV cultures. Two separate experiments (A,B) with 60 minute drug exposures at 10mM resulted in clear reductions in subsequent culture area growth relative to both the 1mM misonidazole and control groups. (XBL 827-4004A)

Figure 5 - Strip chart record of the instantaneous action potential firing frequency of a cerebellar Purkinje cell (19 DIV) before, during, and after exposure to 1mM misonidazole under aerobic conditions. Inserts show samples of isolated single unit activity at times indicated by arrows. Each time division on the inserts is 100 milliseconds and the amplitude eivisions are 100 microvolts. BSS = glucose-fortified balanced salt solutions. See text for further explanation. (XBL 827-10807)

Figure 6 - Instantaneous firing frequency of a 15 DIV cerebellar Purkinje cell before, during, and after sequential exposures (with return-to-control) to a hypoxic insult (dissolved pO_2 = 1.4 ppm), and to two successive pulses of 100 uM misonidazole under aerobic conditions. See text for further explanations. (XBL 827-10808)

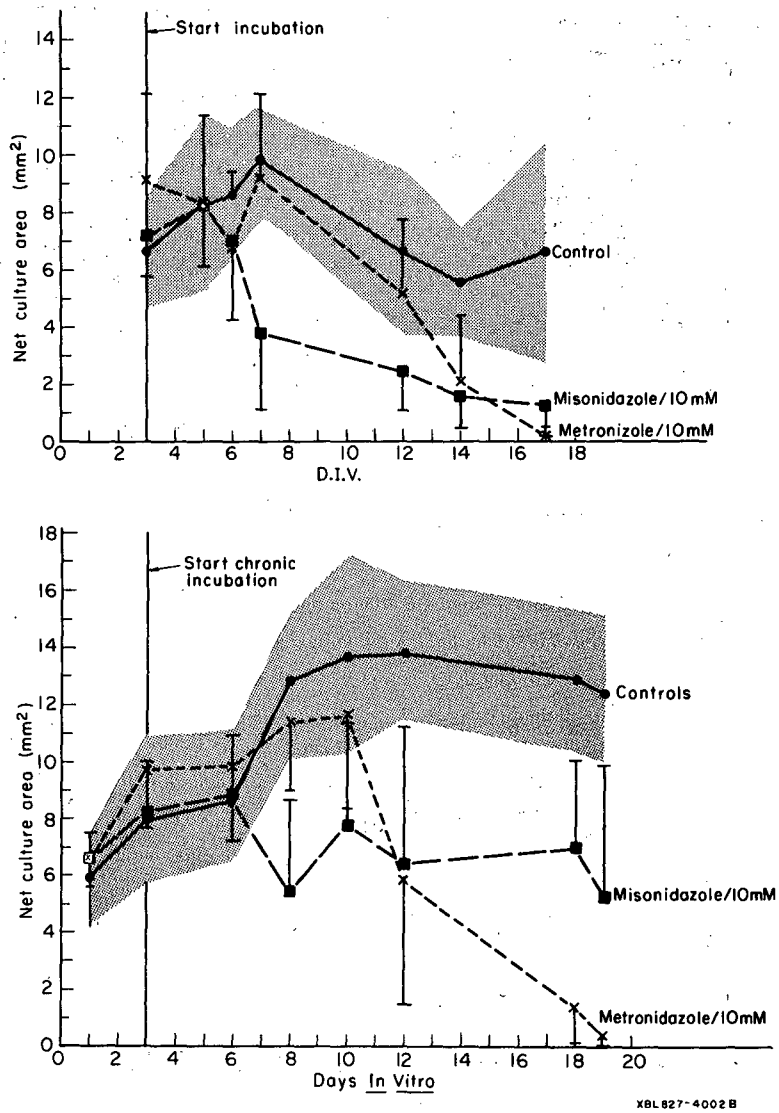
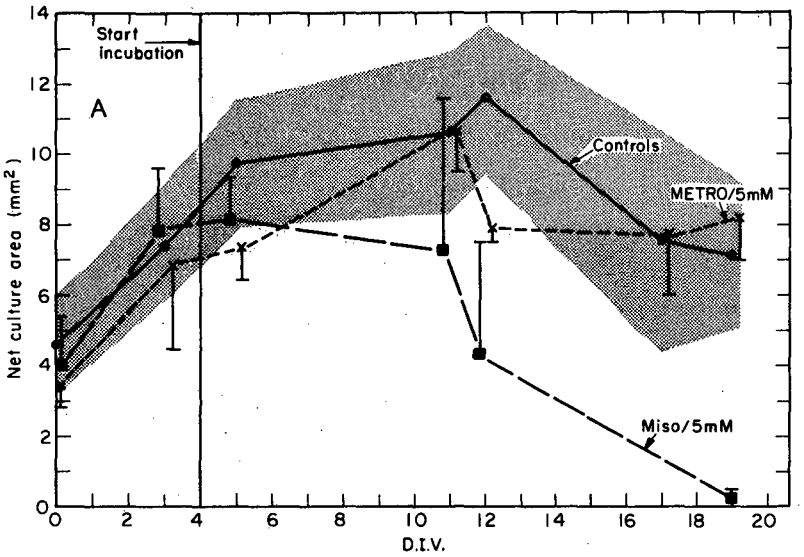

Start incubation
Net culture area (mm²)
Control
Misonidazole/10mM
Metronizole/10mM
D.I.V.
Start chronic incubation
Controls
Misonidazole/10mM
Metronidazole/10mM
Days In Vitro
XBL827-4002B

FIGURE 1


Start incubation
A
Controls
METRO/5mM
Miso/5mM
Net culture area (mm²)
D.I.V.

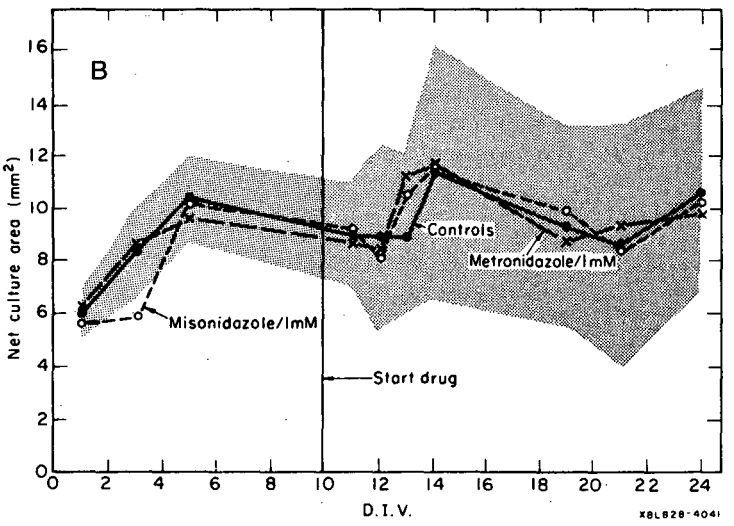

B
Controls
Metronidazole/1mM
Misonidazole/1mM
Start drug
Net culture area (mm²)
D.I.V.
X8L828-4041

FIGURE 2

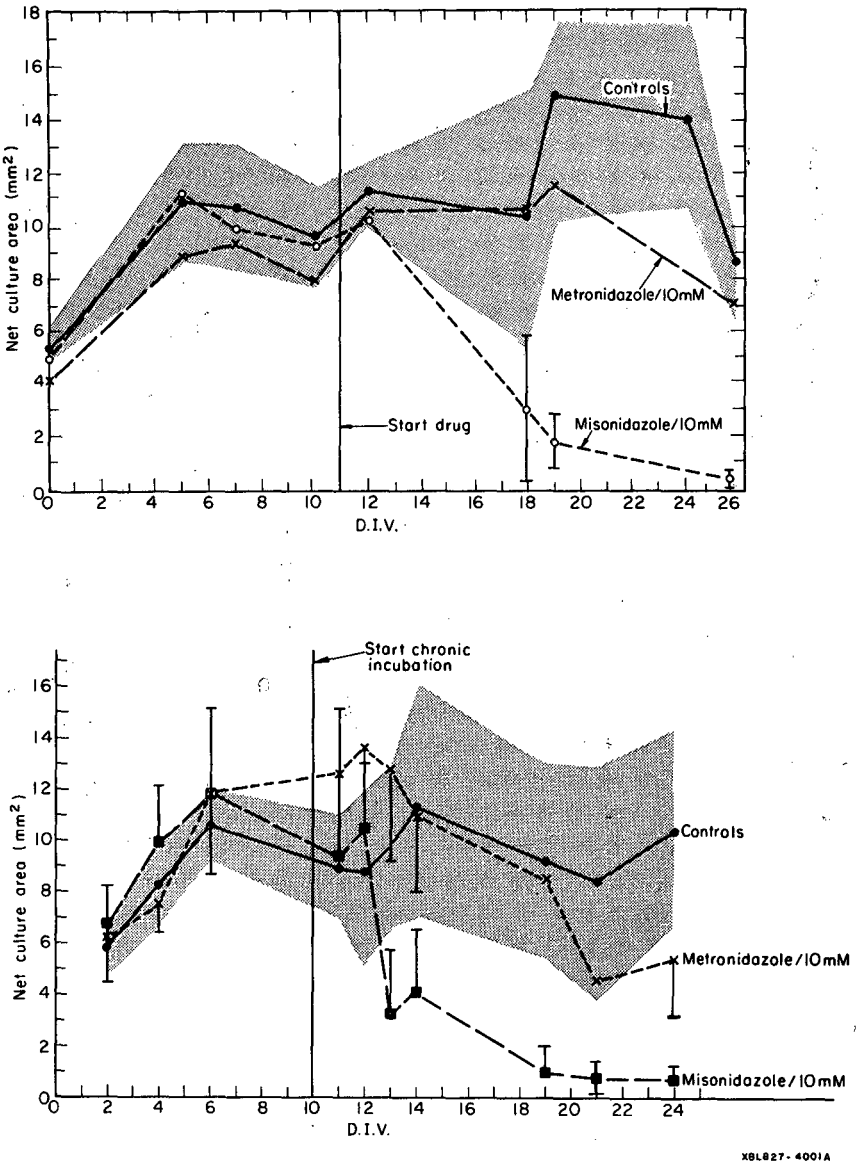

Net culture area (mm²)
D.I.V.
Controls
Metronidazole/10mM
Misonidazole/10mM
Start drug
Start chronic incubation
XBL827-4001A

FIGURE 3

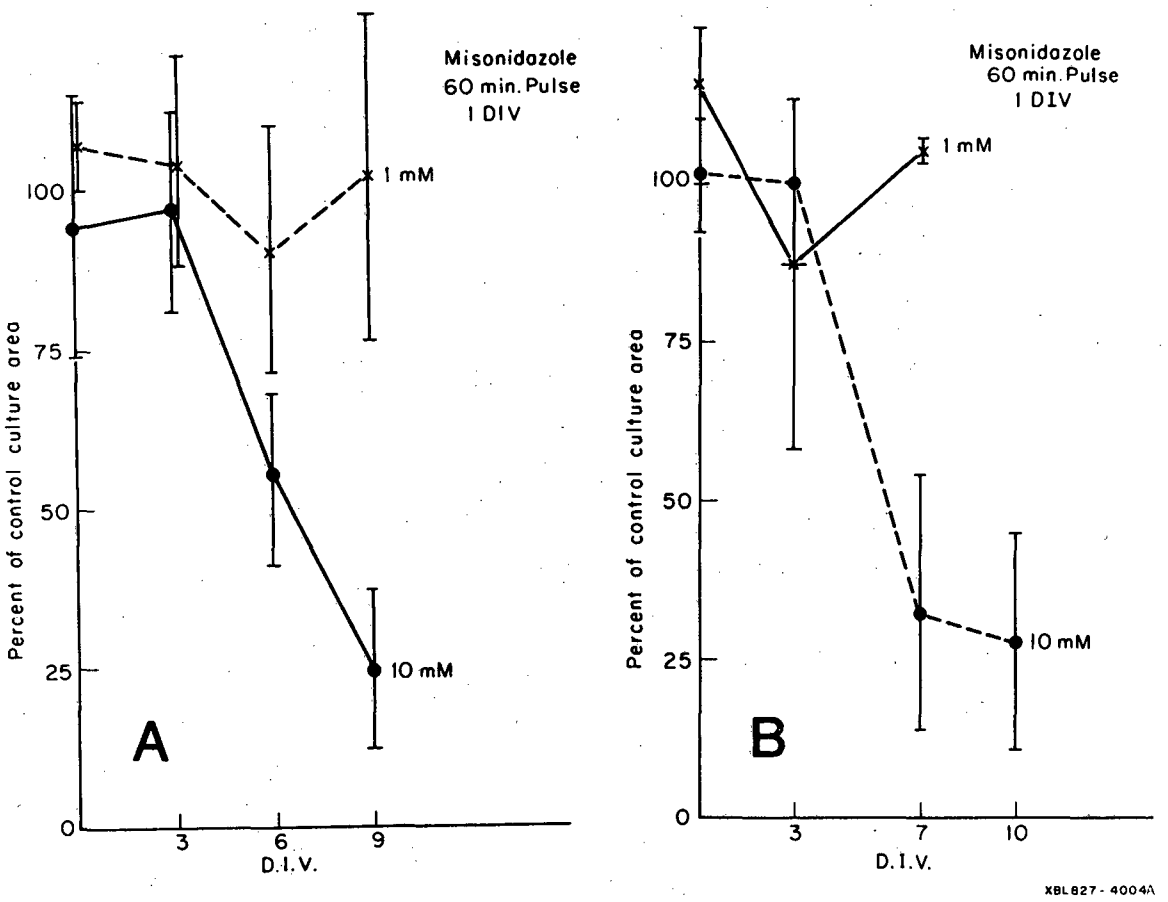
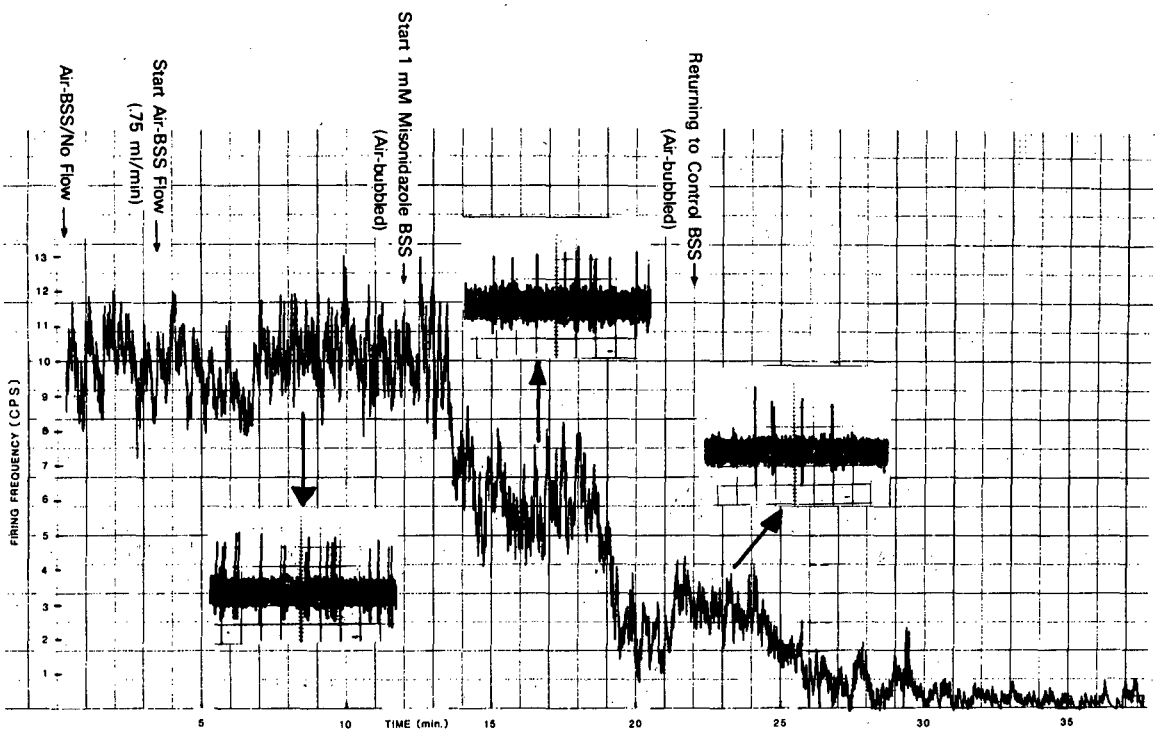

Misonidazole
60 min. Pulse
1 DIV
1 mM
10 mM
A
Percent of control culture area
100
75
50
25
0
3
6
9
D.I.V.
Misonidazole
60 min. Pulse
1 DIV
1 mM
10 mM
B
Percent of control culture area
100
75
50
25
0
3
7
10
D.I.V.
XBL827-4004A

FIGURE 4



XBL 827-10807

FIGURE 5

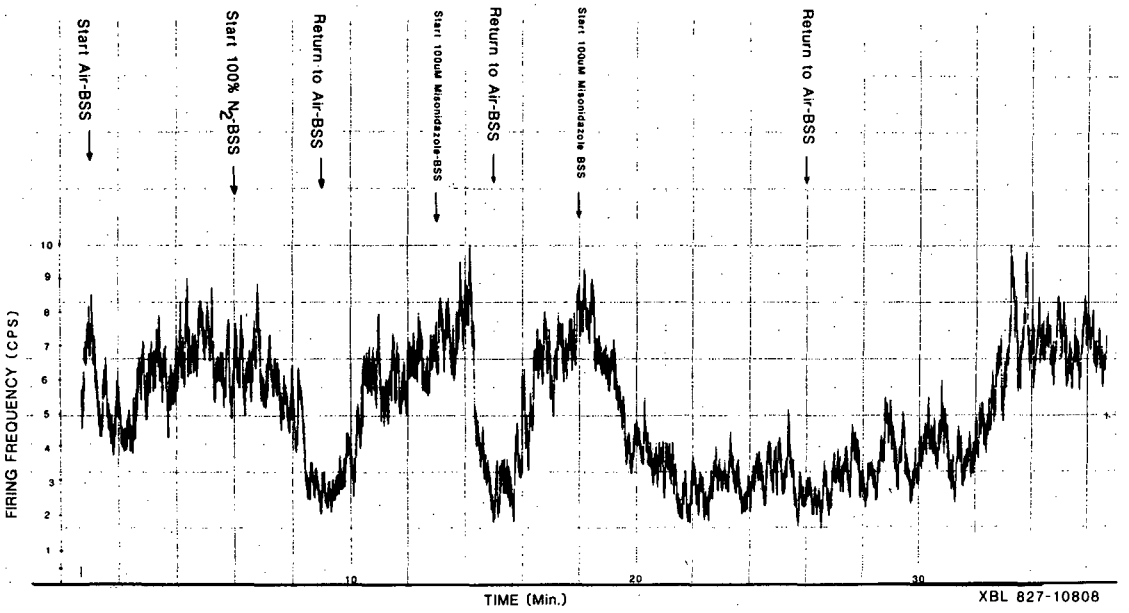

Start Air-BSS
Start 100% N_2-BSS
Return to Air-BSS
Start 100uM Miconidazole-BSS
Return to Air-BSS
Start 100uM Miconidazole BSS
Return to Air-BSS
FIRING FREQUENCY (CPS)
10
9
8
7
6
5
4
3
2
1
10
20
30
TIME (Min.)
XBL 827-10808

FIGURE 6

		D.I.V.					
		10	11	16	18	19	20
24 Hour Pulse	Metronidazole (10mM)	10.27mm^2 ±1.57	13.33 ±1.44	11.86 ±1.08	8.32 ±1.67	12.60 ±3.97	15.52 ±2.78
	Misonidazole (10mM)	11.75 ±1.94	11.32 ±4.96	7.15 ±3.63	8.36 ±3.91	4.94 ±2.29	3.41 ±2.62
48 Hour Pulse	Metronidazole (10mM)	12.10 ±1.70	14.90 ±1.60	7.93 ±2.09	10.75 ±0.64	8.78 ±4.53	16.19 ±3.14
	Misonidazole (10mM)	11.37 ±2.77	9.36 ±3.60	6.75 ±3.12	2.65 ±1.96	1.53 ±1.47	3.68 ±3.04
Controls		10.72 ±2.64	12.12 ±4.00	7.97 ±3.50	7.48 ±3.53	10.67 ±3.56	13.39 ±3.71

TABLE I

XBL828-4040

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