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Effects of the Analgesic Acetaminophen (Paracetamol) and its para-Aminophenol Metabolite on Viability of Mouse-Cultured Cortical Neurons

Stephen Schultz[1], Mauris DeSilva[1], Ting Ting Gu[2], Mei Qiang[2] and Kyumin Whang[2]

[1]Naval Medical Research Unit San Antonio, San Antonio, TX, USA, and [2]University of Texas Health Science Center, San Antonio, TX, USA

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Abstract: Acetaminophen has been used as an analgesic for more than a hundred years, but its mechanism of action has remained elusive. Recently, it has been shown that acetaminophen produces analgesia by the activation of the brain endocannabinoid receptor CB1 through its para-aminophenol (p-aminophenol) metabolite. The objective of this study was to determine whether p-aminophenol could be toxic for *in vitro* developing mouse cortical neurons as a first step in establishing a link between acetaminophen use and neuronal apoptosis. We exposed developing mouse cortical neurons to various concentrations of drugs for 24 hr *in vitro*. Acetaminophen itself was not toxic to developing mouse cortical neurons at therapeutic concentrations of 10–250 μg/ml. However, concentrations of p-aminophenol from 1 to 100 μg/ml produced significant ($p < 0.05$) loss of mouse cortical neuron viability at 24 hr compared to the controls. The naturally occurring endocannabinoid anandamide also caused similar 24-hr loss of cell viability in developing mouse cortical neurons at concentrations from 1 to 100 μg/ml, which indicates the mechanism of cell death could be through the cannabinoid receptors. The results of our experiments have shown a detrimental effect of the acetaminophen metabolite p-aminophenol on *in vitro* developing cortical neuron viability which could act through CB1 receptors of the endocannabinoid system. These results could be especially important in recommending an analgesic for children or individuals with traumatic brain injury who have developing cortical neurons.

Recently, two independent groups have shown that acetaminophen produces analgesia by potentiating endocannabinoids in the brain [1] and that acetaminophen is metabolized in the liver to p-aminophenol which acts in the brain as an indirect agonist at cannabinoid receptors [2,3].

The endocannabinoid system plays an important role in the development of the central nervous system, and its activation can induce long-lasting functional alterations [4]. Use of cannabis (an exogenous cannabinoid) in the still-maturing brain may produce persistent alterations in brain structure and cognition [5]. Animal models have revealed the danger of both cannabis abuse and exposure to cannabinoid drugs during brain development [6], and recently, it has been shown that the naturally occurring endocannabinoid anandamide can cause dose-dependent apoptosis in a human neuroblastoma cell line [7]. Yet, acetaminophen has been frequently used as an analgesic without a doctor's advice to treat fever in children older than 3 months [8], and other safety issues such as liver and kidney damage because of overdose [9], and its effect on embryonic neurons and brain development are unknown.

Peak plasma concentrations of acetaminophen after recommended oral doses range from 8 to 32 μg/ml [10]. At plasma concentrations of <60 μg/ml, acetaminophen does not bind to plasma proteins, and even at 90 μg/ml binding to plasma proteins is less than 5% [11]. Furthermore, acetaminophen is uniformly distributed in most bodily fluids with a tissue/plasma concentration of one [12], and in the cerebral spinal fluid, the plasma partition coefficient is even higher with an estimate of 1.18 [13]. Thus, acetaminophen is readily available not only for its analgesic effect but also for any potential damage to a developing brain.

The purpose of this study was to determine whether treatment with acetaminophen or its metabolite, p-aminophenol, may cause developing neuronal cell death *in vitro* using embryonic mouse cortical neuronal cultures. Our hypothesis is that the acetaminophen metabolite p-aminophenol acting as an indirect agonist of cannabinoid receptors would cause apoptosis similarly to the naturally occurring cannabinoid anandamide.

Materials and Methods

Cortical neuronal cell culture. Primary cortical neurons were prepared from C57BL/6 mouse (Jackson Laboratory, Bar Harbor, ME, USA) as described previously [14]. In brief, cells were dissociated by trypsin treatment and trituration from cortices of foetal mice at embryonic day 15 (E15). Cells were resuspended in minimal essential medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA), 5% heat-inactivated horse serum (Gibco, Auckland, New Zealand), 100 μM L-glutamine (Invitrogen), 28 mM D-glucose and 1X antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA). Cells were plated onto 24-well plates pre-coated with 50 μg/ml poly-L-lysine at a density of ~10,000 cells/well and incubated under 5%

Author for correspondence: Stephen Schultz, Naval Medical Research Unit San Antonio, 3650 Chambers Pass, Ft. Sam Houston, TX 78234, USA (fax 210-916-9311, e-mail stephen.t.schultz@amedd.army.mil).

CO_2 at 37°C. From day 3 onwards *in vitro* (DIV), cells were switched to a serum-free medium system consisting of Neurobasal medium, supplemented with B-27, 100 μM glutamine and 1X antibiotic–antimycotic solution. As Neurobasal medium and B-27 inhibit proliferating glia, the cultures contain >92% neurons.

Drugs. Neuron cultures were then treated with known concentrations of anandamide (Sigma), acetaminophen (Sigma) and the acetaminophen metabolite p-aminophenol (Sigma) at the following concentrations: (i) Anandamide (diluted from 920 mg/ml in 100% ETOH): 100, 50, 1 μg/ml, (ii) acetaminophen (diluted from 50 mg/ml in 100%ETOH): 100, 50, 5, 1 μg/ml. (iii) p-aminophenol (diluted from 1 mg/ml in culture medium): 100, 50, 25, 5, 1 μg/ml, 500 ng/ml.

Cell viability assay. To determine the toxicity level, the viability of neurons as measured by counting cells labelled with fluorescein diacetate and propidium iodide (Sigma) at 24 and 48 hr after administration of the drugs was compared. Statistical significance was determined using analysis of variance (ANOVA) with Neuman–Keuls *post hoc* analysis to assess differences between groups and with the two-sample t-test.

Microscopy. Fluorescent images were obtained using a Nikon TS-100 microscope equipped for both light and fluorescent microscopy, with fluorescence filters for fluorescein and rhodamine. Images were acquired using a CCD camera controlled via NIS FreeWare 2.10 software. Images were obtained with 10X objective lens. Images were collected on each well of a 24-well plate, and viability was measured as green (live cells)/green (live cells) + red (dead cells) × 100.

Results

Effects on cell viability owing to treatment with acetaminophen and p-aminophenol were investigated with E15 mouse cortical neuronal cultures after 3 days *in vitro*. As shown in fig. 1, the viability was significantly reduced for neurons treated with 100, 50, 25, 5 and 1 μg/ml concentrations of p-aminophenol as compared to the controls. The viability did not significantly change in the acetaminophen-treated group (1, 5, 50, 100 and 200 μg/ml) or in neurons treated with 0.5 μg/ml concentration of p-aminophenol as compared to controls. Fig. 2 shows the toxic effect of anandamide treatment compared to controls. The effect of anandamide treatment on mouse cortical neurons is similar to the effect of p-aminophenol.

Representative cortical neuron photomicrographs at 24 hr for control, acetaminophen treated at 100 μg/ml, p-aminophenol treated at 100 μg/ml and p-aminophenol treated at 500 ng/ml are shown in fig. 3.

The toxic effect of p-aminophenol was more pronounced at 48 hr of treatment. No live cortical neurons were present in the cell cultures with concentrations of 1 to 100 μg/ml p-aminophenol at 48 hr (data not shown).

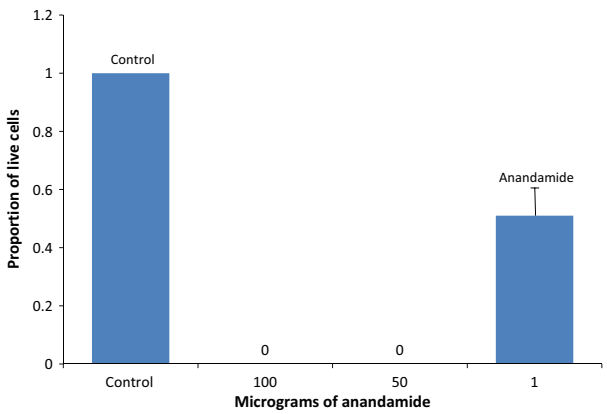

Fig. 2. Comparison of mouse embryonic cortical neuron cell viability after 24-hr treatment with various concentrations of anandamide Anandamide concentrations at 1, 50 and 100 μg/ml showed a significant reduction in the proportion of live cells ($p < 0.05$ by two-sample *t*-test). Error bar is +S.D.

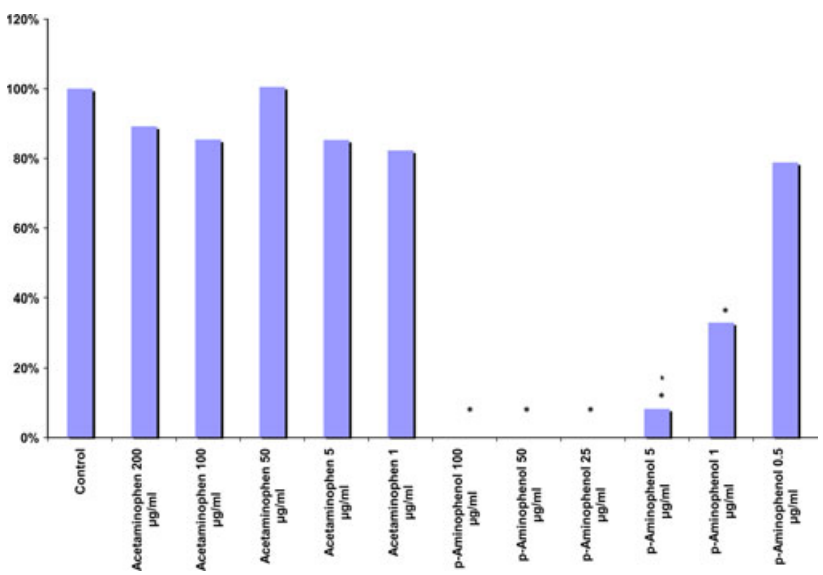

Fig. 1. Comparison of mouse embryonic cortical neuron cell viability after 24-hr treatment with various concentrations of acetaminophen or its p-aminophenol metabolite. *Significantly different from control at $p < 0.001$ by ANOVA with Neuman–Keuls *post hoc* test.

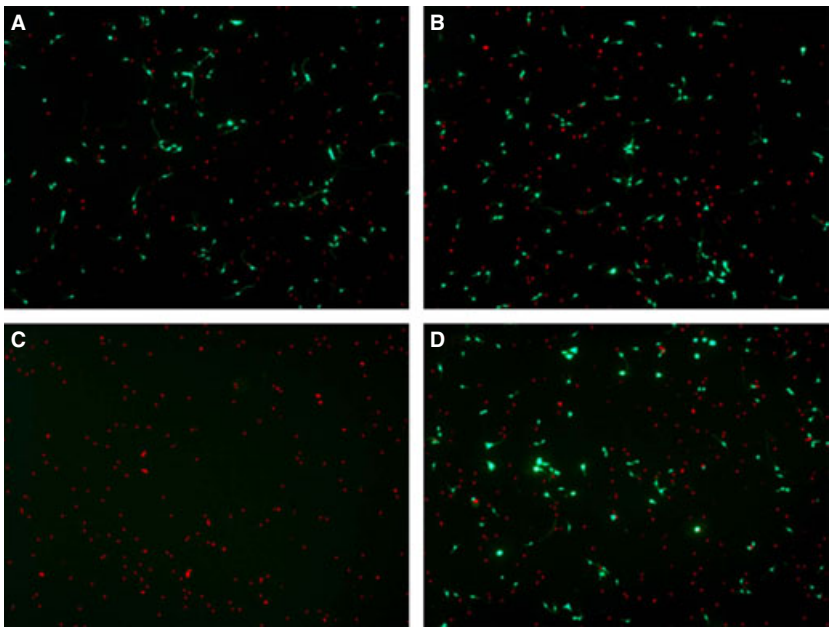

Fig. 3. Live (green) dead (red) fluorescence images of E15 mouse cortical neuron cell cultures treated for 24 hr with (A) control, (B) acetaminophen at 100 μg/ml, (C) p-aminophenol at 100 μg/ml and (D) p-aminophenol at 500 ng/ml.

Discussion

Acetaminophen is a commonly administered analgesic drug, and the results of our experiments have shown a detrimental effect of the acetaminophen metabolite p-aminophenol on *in vitro* cortical neuron viability. The results were similar when testing anandamide, which is an endogenous activator of the endocannabinoid system. To our knowledge, this is the first study to show the toxic effect of p-aminophenol on developing cortical neurons.

We are interested in exploring the safety of acetaminophen, because our previous epidemiological studies linked acetaminophen use with increased risk of autism and suggested that the mechanism of action may be activation of the endocannabinoid system [15–17]. We have previously found that acetaminophen use at age 12–18 months increased the odds of autism in our sample by more than eight times, and by more than 20 times when considering only children who experienced normal development followed by a regression in development. One of the primary features of autism is abnormal brain development [18]. We are suggesting that the abnormal brain development seen in autism may be due to the use of acetaminophen at a critical early age.

Clinicians may need to be more cautious in the recommendation for use of acetaminophen until its safety for developing neurons in children can be verified.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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