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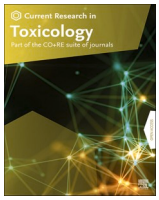

Effect of vinpocetine on embryonic heart rate *in vitro*

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ABSTRACT

Vinpocetine is a readily available nutritional supplement claimed to improve memory and weight loss. However, it blocks the *I*kr current essential for cardiac action potential repolarisation and Ikr inhibition can cause "torsade de pointes" arrhythmias and sudden death. Moreover, Ikr blockers have exhibited teratogenic effects in reproductive toxicology studies, leading to increased birth defects and embryonic mortality. The FDA advises against vinpocetine use in pregnant and prospective mothers based on animal studies showing dose-dependent fetal mortality in rats and rabbits, and cardiovascular malformations in surviving fetuses. However, the mechanisms responsible for vinpocetine's fetal toxicity remain unclear.

The present study used rat embryo culture to evaluate vinpocetine and its major metabolite, apovincaminic acid, on embryonic heart rate, a possible causative factor behind its adverse effects. Both compounds induced embryonic bradycardia in a concentration-dependent manner, with vinpocetine proving more potent.

The minimum vinpocentine concentration to induce bradycardia was 100 nM, a level unlikely to be reached in humans following typical doses. Embryonic arrhythmias were also observed at the highest concentrations.

These results suggest that the FDA's cautionary statement may generate undue anxiety, although re-evaluation of teratogenicity risk associated with vinpocetine should be revisited if a link to cardiac arrhythmias in adults is established.

Introduction

Vinpocetine is a semi-synthetic derivative of vincamine, an alkaloid obtained from the periwinkle plant. It is claimed to improve cerebral perfusion thereby improving the oxygen supply of the brain. The suggested doses recommended by the Physicians' Desk Reference for Nutritional Supplements and the doses that are suggested on available product labels range from 5 to 90 mg/day (Hendler and Rorvik, 2001). In some European countries vinpocetine (Cavinton ®) is regulated as a prescription drug (French et al., 2016), although it is also available without prescription on the internet where it is advertised for a wide range of conditions including weight loss and improving memory.

In June 2019, the FDA issued a statement advising pregnant women and women who could become pregnant not to take vinpocetine (US Food and Drug Administration, 2019). This advice was based primarily on a draft report by the National Institute of Health's National Toxicology Program (NTP), asserting that consumption of vinpocetine is associated with adverse reproductive effects in rats and rabbits (National Toxicology Program, 2019). A final report was subsequently issued (National Toxicology Program, 2020). In pregnant rats and rabbits, vinpocetine caused a dose-dependent increase in fetal death (Catlin et al., 2018). Moreover, in rats some of the surviving fetuses showed cardiovascular malformations, although this was not examined in rabbits.

If vinpocetine really is a risk in human pregnancy the FDA warning statement may be inadequate. About 50% of pregnancies are unplanned (Office of Disease prevention and Health promotion, 2020) and awareness of pregnancy may not occur until well into the organogenic period of development (Branum and Ahrens, 2017). If a woman waits until she is aware that she is pregnant before stopping or avoiding the consumption of vinpocetine it may well be too late to avoid adverse fetal effects. It is proposed that a better understanding of the mechanism by which vinpocetine causes adverse pregnancy in animals may help to determine the risk to human development and the adequacy or necessity of warning statements.

The combination of cardiovascular malformations and increased fetal death in the animal studies suggests that vinpocetine may interfere with the normal functioning of the early embryonic heart. It has been shown previously in rat studies that reduced heart rate and/or abnormal cardiac rhythm during early embryonic development can result in

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abnormal embryonic development causing heart and vascular malformations (Sköld et al., 2001) and, in some cases, embryonic death, possibly due to increased hypoxia (Ritchie et al., 2015). Vascular and cardiac development rely on normal blood flow, and cardiac malformations have been demonstrated when there is a reduction in fetal blood flow or an abnormal flow pattern (Midgett et al., 2017). Severe or prolonged reduction in blood flow leads to death of the embryo (Franklin and Brent, 1964; Leist and Grauwiler, 1974).

In the human the hERG potassium channel protein plays an important role in cardiac muscle repolarisation and is expressed in both the embryo and the adult. However, in the rat its expression is limited to the early embryo and it is not detected in the adult rat (Danielsson et al., 2013). This developmental stage of the rat embryo, and by extension, in the human embryo, is particularly vulnerable to drug-induced cardiac arrythmias. Studies on pregnant rats have shown that exposure to various hERG blockers cause bradycardia and cardiac arrhythmias in early rat embryos, resulting in malformations and increased embryonic mortality (Webster et al., 1996; Sköld et al., 2001; Sköld et al., 2002).

Vinpocetine has been reported to block the Ikr channel with an IC 50 of 130 nM (Yunomae et al., 2007). This is lower than the reported average plasma C_{max} of vinpocetine in humans of which ranges from 57 nM to 191 nM (average 183.5 nM) (Vereczkey et al., 1979; Abd Elbary et al., 2002). These findings suggest a potential mechanism of how vinpocetine may cause fetal adversity is through its effects on embryonic heart rate. However, to date there are no reports on the effects of vinpocetine on embryonic heart rate.

In the present study the effect of different concentrations of vinpocetine and its main metabolite apovincaminic acid (Miskolczi et al., 1990) on the functioning of the rat embryonic heart was determined using cultured gestational day (GD) 13 rat embryos which correspond to 5–6 week human embryos (Sissman, 1970).

Materials & methods

Animals

The University of Sydney Animal Ethics Committee approved all animal work in this study. Sprague-Dawley (SD) rats (Animal Resources Centre, Murdoch, Western Australia) were group-housed under a 12:12 h light/dark cycle at 22–26 °C and 40–60% humidity with free access to standard rodent chow and water. Female rats were mated overnight and examined the next morning for a sperm-positive vaginal smear. Mated rats were separated and considered to be GD0.

Embryo culture

On GD13 the pregnant dams were anaesthetized by CO_2 inhalation and killed by cervical dislocation. The uterus was removed, washed in 0.1 M phosphate buffered saline (Sigma-Aldrich, St. Louis, MO), opened and decidua removed. Reichart's membrane was removed leaving the embryo surrounded by an intact yolk sac. A small incision was made in the yolk sac, avoiding damage to major blood vessels, the amnion was then opened, and the embryo was eased through the incision. Each embryo was then transferred to a culture bottle containing 2.5 mL of Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) and placed in a rotating culture chamber (11 rotations per minute) where they were continuously gassed with 95% O_2 and 5% CO_2 and maintained at 38 °C.

After 1 h in the incubator each culture bottle, housing a single embryo, was placed on a heated stage that maintained culture fluid temperature at 38 °C and the embryo was examined under a dissecting microscope (Leica M 420, Leica Microsystems Ltd., Heerbrugg, Switzerland) equipped with a video recorder (Olympus DP70, Olympus Australia Pty Ltd., Melbourne, Australia). Damaged embryos were rejected at this stage. The embryonic heart was clearly visible through the neck of the open culture bottle and videos of the embryos were recorded for 20 s (30 frames/sec). Embryos were then randomly assigned to treatment or control group. Previous studies have shown that addition of 25 μM dimethyl sulfoxide (DMSO) does not significantly affect the embryonic HR (EHR) (Ritchie et al., 2019). Each culture bottle with its single embryo was then administered 25 μL of test chemical (vinpocetine or apovincaminic acid at varying concentrations) or DMSO vehicle and then replaced in the incubator for an additional 1 h incubation. The embryo was then returned to the microscope stage and for a second 20 s video recording before the embryos were euthanised by decapitation.

Chemicals

Stock solutions of vinpocetine (Focus Bioscience, St Lucia, Qld), and apovincaminic acid (Focus Bioscience, St Lucia, Qld) were prepared by dissolving in DMSO with aliquots stored at −20 °C. Serial dilutions were prepared on the day of the experiment from stock solutions.

Concentration range studies

To establish the lowest observed effect concentration (LOEC), vinpocetine was tested at final concentrations of 0.1, 1, and 5 μM. Apovincaminic acid was tesed at concentrations of 25, 50, 100 and 200 μM. Each embryo was exposed to as single chemical concentration. Except for apovincaminic acid (which was limited by availability), a minimum of ten embryos were tested at each concentration.

Image analysis of cardiac effects

Embryos with a heart rate of $\leq$ 180 beats per minute after the first hour in culture (pre-treatment) were considered damaged and not used in the experiment. Video recordings of the embryonic hearts were analyzed using software developed at the University of Uppsala (Khan et al., 2008). The atria and ventricles of the heart were identified on the video image and were manually marked. The program measures light–dark intensity changes on a relative scale (0–1) in the selected area of the heart over a 15 s period. Traces of atrial and ventricular activity were created by the image analysis program with one (1) representing a filled chamber (end-diastole) and zero (0) representing an emptied chamber (end-systole). The period between successive troughs was taken as the interbeat interval, and the average interbeat interval was used to determine EHR (beats per minute).

Statistics

After analysis of the videos, EHR was classified as regular, irregular, or arrhythmic. Irregular heart beats showed alternate atrial and ventricular contractions with variable interbeat intervals. Arrhythmic heart beats demonstrated loss of synchrony between atrial and ventricular contractions and partial contractions of the atria and ventricles and were unable to be counted. Dead (asystole) embryos and those with arrhythmias were not included in the statistical analysis of EHRbut noted in the results.

All data are expressed as mean ± SEM and 95% confidence intervals. Heart rates before and after drug were compared using a paired *t*-test (IMB 153 SPSS Version 19 software). In all tests, significance was taken as $p < 0.05$. The lowest concentration that produced a significant change in EHR was classified as the lowest observed effect concentration (LOEC).

Results

GD 13 rat embryos survive well in the culture conditions used in this study and maintain a heart rate of over 220 beats per minute (bpm) with a regular rhythm. The addition of vinpocetine caused a concentration-dependent decrease in EHR ranging from over 50% reduction at 5 μM

to ~9% reduction at 100 nM (LOEC; Table 1; Fig. 1). One embryo at 1 μM had an irregular heart rhythm (Fig. 1) and two embryos at 5 μM showed arrhythmias.

Exposure to apovincaminic acid also caused a concentration-dependent decrease in EHR with an LOEC of 50 μM (Table 1) with no heartbeat irregularities observed.

Discussion

Effect of vinpocetine on embryonic heart

The GD 13 rat embryo used for this study is similar in size and cardiovascular development to a 5–6-week-old human embryo (Sissman, 1970). At this stage of development, both the human and rat embryo rely on the hERG potassium current for repolarisation (Danielsson et al., 2013) (Nilsson et al., 2013). Exposure to drugs known to block the hERG *I*kr channel consistently cause bradycardias and arrythmias in rat embryos *in vitro* (Webster et al., 1996; Sköld et al., 2001; Sköld et al., 2002; Abela et al., 2010). In this study, vinpocentine also induced embryonic bradycardia at concentrations consistent with *I*kr patch-clamping studies that demonstrated an IC 50 of 130 nM for vinpocetine blockade (Yunomae et al., 2007).

The main metabolite of vinpocetine in rats and humans is apovincaminic acid (Vereczkey and Szporny, 1976; Miskolczi et al., 1990) reaching up to 4.2 μM in the rat (Vereczkey and Szporny, 1976) and 0.082 μM in the human (Vlase et al., 2005). There was no effect on embryonic heart rate *in vitro* up to 200 μM. It is unlikely that the metabolite is the proximate cause of the observed embryonic bradycardic response to vinpocetine.

Drugs that inhibit the hERG channel have often shown teratogenic effects in rat reproductive toxicology studies causing an increase in birth defects and embryonic death (Webster et al., 1996; Sköld et al., 2001; Sköld et al., 2002). This might suggest a mechanism of action for the positive developmental toxicology studies of vinpocentine (Catlin et al., 2018). However, there remains some doubt about the relevance of these results for the human. This is because while the hERG potassium current is important for repolarisation in the adult human, it less important in the adult rat (Danielsson et al., 2013). A consequence of this difference is that pregnant rats can tolerate high doses of hERG inhibitors that affect the embryo but have no observable effect on the dam. In contrast, in humans, similar concentrations would likely induce adverse side-effects limiting exposure and rendering interpretation of *in vivo* studies problematic. This disparity between species highlights the need for caution when extrapolating findings from animal studies to potential effects in humans.

Table 1
Effect of Vinpocetine and Apovincaminic Acid on Rat Embryonic Heart Rate *In Vitro*.

Chemical (No. embryos)	No. embryos Irregular or arrhythmic EHR	pre-EHR (beats/min) mean ± SEM	post EHR (beats/min) mean ± SEM	EHR change (%) mean ± SEM	95% CI of mean EHR change
Vinpocetine					
DMSO (16)	0	231.5 ± 3.69	230.8 ± 3.26	−0.11 ± 1.51	−3.1 to 3.1
100 nM (11)	0	230.6 ± 7.8	208.7 ± 6.17	−9.1 ± 1.73*	−13.0 to −5.3
1 μM (10)	1	232.8 ± 5.71	142.8 ± 10.75*	−38.7 ± 4.18*	−48.2 to −29.3
5 μM (10)	2	228.0 ± 4.26	104.0 ± 9.30*	−54.7 ± 3.60*	−62.8 to −46.6
	Apovincaminic Acid				
DMSO (9)	0	227.1 ± 4.61	233.3 ± 5.08	3.0 ± 2.78	−3.4 to + 9.4
25 μM (10)	0	232.8 ± 4.17	233.2 ± 3.68	0.2 ± 0.97	−2.0 to + 2.4
50 μM (11)	0	235.3 ± 2.74	217.1 ± 5.42	−7.7 ± 2.06*	−12.3 to −3.1
100 μM (10)	0	231.6 ± 3.98	195.6 ± 4.75*	−15.4 ± 2.16*	−20.3 to −10.5
200 μM (5)	0	232.0 ± 3.10	154.8 ± 3.71*	−31.7 ± 1.45*	−35.7 to −27.7

Risk assessment

The FDA pregnancy warning (US Food and Drug Administration, 2019) asserts that the blood levels of vinpocetine measured in positive pregnant animal studies were similar to those reported in humans after taking a single dose of vinpocetine. The significance of these findings hinges on the importance placed on the developmental toxicology results and extrapolation of laboratory animal pharmacokinetic studies.

In the NTP teratology study, rats were dosed with 0, 5, 20 or 60 mg/kg/d of vinpocetine from GD 6 to 20 (Catlin et al., 2018). The authors concluded that embryotoxicity was observed at the lowest dose of 5 mg/kg, based on an increased incidence of ventricular septal defects, although this has been disputed as being within the normal range of historical controls (Wise et al., 2018). The lack of a proposed mechanism was also crticised.

The present study offers a plausible mechanism. If vinpocetine concentration exceeds the observed threshold (100 nM) *in vitro* then it would be expected that embryonic bradycardia and possible arrythmias would be induced. If bradycardia persists, as has been observed with other hERG blocking agents, then fetal malformation or death would be expected (Ritchie et al., 2015).

Whole embryo culture demonstrated that exposure to a threshold concentration of 100 nM vinpocetine produced a ~10% fall in EHR. However, since the embryos are cultured in a protein free media (DMEM) it is more appropriate to compare the lowest effect concentration with "free drug" plasma concentrations in the human. Protein binding of vinpocetine in human plasma has been reported to be concentration-dependent and ranges from 86.6% at 1427 nM, 97.8% at 285 nM and 99.9% at 28.5 nM (Polgár et al., 1985). This has significant implications for estimating the margin of safety. Although there are no available data on the degree of protein binding of vinpocetine in the rat, if it is similar to the human then the estimated free vinpocetine concentration at both 5 mg/kg and 20 mg/kg (Waidyanatha et al., 2018) would exceed the threshold observed *in vitro* (Polgár et al., 1985) (Table 2). Therefore, the *in vitro* data support the claims in the NTP teratology study that a 5 mg/kg dose may be embryotoxic (Catlin et al., 2018). Moreover, the observed embrytoxicity and cardiovascular defects could be explained by embryonic bradycardia, as reported in the current and earlier studies (Webster et al., 1996; Sköld et al., 2001; Sköld et al., 2002), and are consistent with the finding that vinpocetine blocks hERG (Yunomae et al., 2007). The resulting disruption in normal blood flow might then contribute to cardiac malformations (Midgett et al., 2017) and, if severe or prolonged, embryonic death (Franklin and Brent 1964, Leist and Grauwiler 1974).

Extrapolation of in vitro results to the human

The embryo culture results can be used to directly extrapolate to the human by comparing the lowest concentration causing a reduction in EHR in the embryo culture system (100 nM) with human plasma levels (Nilsson et al., 2010). Human exposure to 10 mg vinpocetine has been associated with plasma Cmax range of 177–191 nM (Abd Elbary et al., 2002) and 57–17913 nM (Kharshoum et al., 2013). At average human exposures, this would indicate a protein-free Cmax of 4.03 nM (using 97.8% binding) and 24.8 fold margin of safety or greater at the lower Cmax levels (Table 2). The margin of safety is even higher if there is a greater degree of protein binding (Polgár et al., 1985). Alternatively,

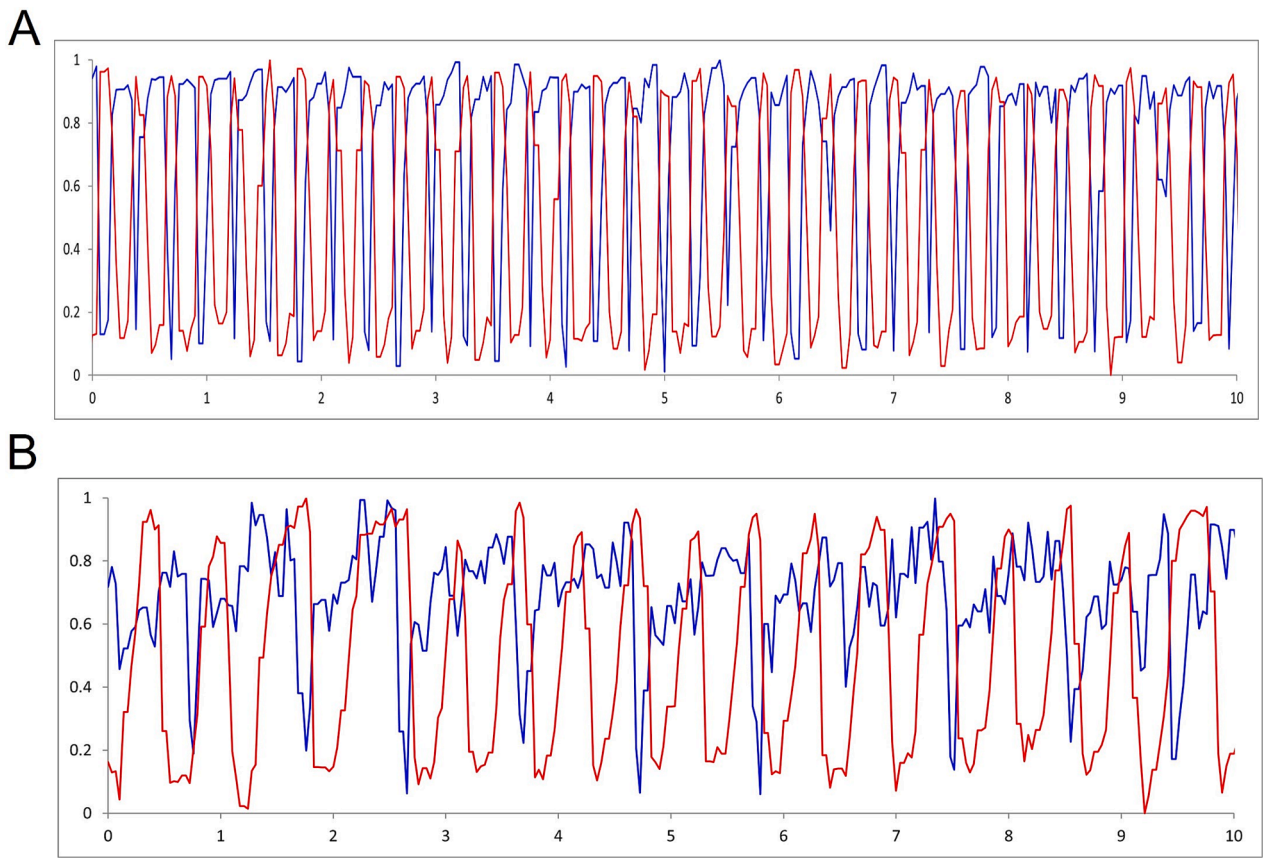

Fig. 1. Ten second traces of the embryonic heart. The trace for the atria is red and for the ventricles blue. (A) Before addition of vinpocetine, heart rate = 224 bpm (B) one hour after addition of vinpocentine 1 μM, heart rate = 100 bpm (based on atrial contraction). In this example, there were approxiametely two atrial contractions for each ventricular contraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Comparison of pharmacokinetic values and margins of safety of vinpocetine in rats and humans using *in vivo* and *in vitro* data.

Dose, duration and species	AUC (0-T) ng/mL·h (nM)	Cmax ng/ml (nM)	Cmax (est unbound[2]) nM
5 mg/kg GD 6–18 rats[1]	1830 (5222 nM)	497 (1418 nM)	190 nM (86.6%)
20 mg/kg GD 6–18 rats[1]	7020 (20031 nM)	1420 (4052 nM)	543 nM (<86.6%)
10 mg single dose human[3]	519.7 (1483 nM)	64.3 (18.5 nM)	24.5 nM (86.6%)

[1] (Waidyanatha et al., 2018) [2] estimated unbound concentration assuming 86.6% and 97.8% protein binding (Polgár et al., 1985) [3](Abd Elbary et al., 2002).

free vinpocetine concentration of 100 nM may only occur in the human at plasma concentrations about 20 times higher or about 3.6 μM, an unlikely scenario that would expected to be associated with adverse cardiovascular maternal effects.

It is is relevant to note, however, that the natural alkaloid, vincamine, has been associated with ventricular arrythmia and torsades de pointes in humans following intravenous and intramuscular injection (Dany et al., 1981). In most instances, these cases were associated with underlying conditions eg. hypokalemia and long QT syndrome. It is clear that pre-existing conditions may decrease the margin of safety considerably.

The calculated margins of safety assume a typical 10 mg dose of vinpocetine. However, higher doses up to 90 mg/day are readily available (Hendler and Rorvik, 2001) and the pharmacokinetic profile at higher doses is unknown but appears to be linear (Miskolczi et al., 1990). The actual concentration in commercially available vinpocetine in the United States varied from of 0.6–5.1 mg/serving (French et al., 2016). As a nutraceutical, vinpocetine is not regulated in the United States, a circumstance that would change if it was regulated and required compliance testing. Finally, as vinpocetine has poor aqueous solublity with reduced bioavailability, alternative delivery vehicles and routes of exposure have been explored with a much higher Cmax reported in rats using a nasal spray delivery (Aldawsari et al., 2022).

Conclusions

The present study suggests a plausible mechanism of action underlying the induced adverse effects associated with vinpocetine in the NTP teratology study underpinning the FDA pregnancy warning (US Food and Drug Administration, 2019). Vinpocetine reduces embryonic heart rate at a free concentration of 100 nM. This effect observed *in vitro* is consistent with the reported property of hERG inhibition by vinpocetine. The embrytoxicity and cardiovascular defects observed in the NTP study could be explained by embryonic bradycardia. However, the estimated free Cmax associated with adverse pregnancy outcomes in rats is unlikely to be reached in humans following a typical single 10 mg dose of vinpocetine. The current FDA warning statement will not prevent accidental exposure in early pregnancy and could potentially cause unnecessary anxiety in exposed pregnant women. As far as we are aware there have not been any reports of birth defects associated with vinpocetine consumption during pregnancy. It is essential to remember that birth defects occur in 2–3% of all pregnancies, and that associations will occur

by chance alone. If cardiac arrhythmias or torsdes de pointes in adults are shown to be associated with vinpocetine consumption, then the possibility of teratogenicity should be revisited.

In conclusion, while the present study provides valuable insights into vinpocetine's effects on the embryonic heart, the overall risk to humans following standard doses appears low. Nevertheless, the potential risks associated with higher doses or alternate delivery methods should not be overlooked, and further investigation may be necessary to ensure safe usage of vinpocetine in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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