

RESEARCH ARTICLE

# Progressive heart P-glycoprotein (P-gp) overexpression after experimental repetitive seizures (ERS) associated with fatal status epilepticus (FSE). Is it related with SUDEP?

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Received: January 21, 2014

Published online: February 19, 2014

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Patients with refractory epilepsy (RE) have increased risk of Sudden Unexpected Death in Epilepsy (SUDEP), where acute and fatal heart failure is suspected. High seizure frequency, polypharmacy, changes in dosing, persistent low AED levels or poor adherence to therapy are the greatest risk factors of SUDEP and are also features observed in RE. We evaluated the progressive P-gp overexpression in heart, related with the development of fatal status epilepticus (FSE) after experimental repetitive seizures (ERS). Male Wistar rats (180–230g) were daily injected (i.p) with Pentylentetrazole (PTZ; 45mg/kg; n=18) or saline (Controls; n=6). Severity of seizures was recorded. Four PTZ-treated rats were sacrificed at 4<sup>th</sup>-7<sup>th</sup> day respectively. Ten remaining rats, underwent same treatment until develop fatal status epilepticus (FSE). Brains and hearts were studied by immunofluorescent method for P-glycoprotein (P-gp) expression. Seizures were observed each day of PTZ treatment, associated with a progressive P-gp overexpression in heart and FSE at 9<sup>th</sup> day. Using the same PTZ model, we previously demonstrated that progressive brain P-gp overexpression contributes to cell membrane depolarization of hippocampus and neocortex. These are the first evidences showing that ERS induces a simultaneous and progressive P-gp overexpression in brain and heart associated with FSE, and suggests a role for this pattern expression of P-gp as risk factor for death during SE. The simultaneous and spontaneous death of all animals during SE observed only at day 9<sup>th</sup>, suggest that a higher P-gp overexpression in cardiomyocytes could play a role in SUDEP, however, because it is only a study descriptive, further researches are needed to confirm this hypothesis.

**Keywords:** P-glycoprotein; repetitive seizures; pentylentetrazol; SUDEP; status epilepticus; myocardium

*Molecular & Cellular Epilepsy* 2014; 1:43-51. doi: 10.14800/mce.66; © 2014 by Smart Science & Technology, LLC.

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## Introduction

Epilepsy carries an increased mortality risk of approximately two or three times the general population. This issue is even more critical in the subgroup of patients with drug-resistant epilepsy, where mortality rate is more elevated than in patients with well-controlled seizures and it has emerged as a major public health issue over the last decade<sup>[1-5]</sup>. Sudden Unexpected Death in Epilepsy (SUDEP) is defined as sudden, unexpected, witnessed or unwitnessed, no traumatic and no drowning deaths in patients with epilepsy, in which post-mortem examination does not reveal a toxicological or anatomical cause of death<sup>[4]</sup>.

Several studies have attempted to identify clinical characteristics of patients with epilepsy at particular risk for SUDEP. Interestingly, long history of epilepsy (defined as more than 15 years), high seizure frequency (defined as more than 15 seizures per month), antiepileptic drug (AED) polypharmacy or frequent changes in dosing, persistent low plasma levels of AEDs or poor adherence to pharmacotherapy, are defined as the greatest risk factors of SUDEP that appears to correlate with the severity of the epilepsy<sup>[5-8]</sup>.

Because several of these features are associated to pharmaco-resistant epilepsy, it is not surprising that SUDEP should be more likely to occur in patients who have developed refractory epilepsy (RE) phenotype<sup>[9-12]</sup>.

A main emerging mechanism to explain RE is the increased brain expression and/or function of multidrug transporter proteins, particularly the P-glycoprotein (P-gp), the product of the multidrug resistance protein 1 (MDR1) gene<sup>[13-16]</sup>.

It has been previously demonstrated that experimental repetitive seizures (ERS) by daily administration of 3-mercaptopropionic acid (3-MP), induce a progressive increase of P-gp brain expression, affecting not only the blood-brain barrier (BBB) cells, but also astrocytes and neurons<sup>[16]</sup>. P-gp expression is increased in BBB vessels and related astrocytes after 4 days of 3-MP treatment, while neurons were highly immunoreactive after the 7<sup>th</sup> 3-MP administration. In this experimental model, animals showed a progressive loss of protective effects of phenytoin (PHT)<sup>[17]</sup>, whereas nimodipine, a P-gp inhibitor, restores the altered hippocampal PHT pharmacokinetics and reverted the refractory phenotype<sup>[18]</sup>. In addition, the continuous daily 3-MP-induced seizures resulted in PHT-refractory fatal status epilepticus (FSE) at day 13<sup>th</sup>, except in animals treated with Nimodipine plus PHT<sup>[19]</sup>.

Although epilepsy and SE are, by definition, not causes of death, seizure activity can induce cardiovascular alterations (severe ictal tachycardia and abnormalities of the heart rhythm or repolarization) and changes in the respiratory rate and pattern including tachypnea, hypopnea or apnea<sup>[20]</sup>, all of them risk factors of heart ischemia<sup>[21]</sup>. The exact pathophysiological causes of SUDEP are unknown but it is very likely that cardiovascular and respiratory abnormalities during and between seizures, play a potential role.

On the other hand, a group of evidence indicates that P-gp can also decrease plasma membrane potential of several cell types<sup>[22,23]</sup> and modulates the swelling-activated chloride currents, both physiologic disturbances observed during brain and convulsive stress<sup>[24-26]</sup>. Recently, we demonstrated that progressive P-gp brain overexpression secondary to repetitive seizures induced by pentylenetetrazol (PTZ) in rats, contributes to an also progressive cell membrane depolarization of hippocampus and neocortex<sup>[27]</sup>. On the other hand, since SUDEP has been related to mechanisms similar to those of sudden cardiac death, it has been suggested that cardiovascular alterations are associated to SUDEP<sup>[28]</sup>.

Based on the mentioned background, we designed experiments to determine if seizures induced after daily PTZ administration are associated to simultaneous and progressive increase of P-gp expression in brain and heart, and FSE.

## Material and methods

PTZ and propidium iodide were obtained from Sigma Chemical (St. Louis, MO). All other chemical substances were of analytical grade.

*a. Animals and PTZ-induced seizures:* Male Wistar rats (n=18), weighing 180–230 g. were maintained for at least 1 week on a 12-h diurnal cycle before use. Then, they were daily i.p. injected with a single dose of PTZ (45 mg/kg dissolved in saline solution). The severity of the seizures was rated according the following criteria: *Grade I:* Clonic seizures of forelimbs and some tonic jerks of hindlimbs; *Grade II:* Tonic-clonic seizures with clearly defined interictal episodes; *Grade III:* Tonic-clonic seizures and SE, characterized by long lasting episode of convulsive activity, usually ending in a tonic hyperextension of all limbs with further recovery; *Grade IV:* SE with very short or absent interictal periods and death.

The latency and duration of the different behavioral changes and incidence of death were daily recorded in each animal. Values of days 1, 4 and 7 were compared.

**Table 1. Latency and time duration of seizures were recorded in seconds.** Values represent the mean  $\pm$  SD from all animals evaluated together. PTZ-1 and PTZ-4 corresponds to the total of 18 rats. PTZ-7 corresponds to 14 animals with severity Grade II (n=11) or III (n=3). Four animals with grade II were sacrificed at 7th day. The remaining alive rats, were treated for longer time (n=10) to reach fatal SE (Grade IV).

	Groups			
	PTZ-1 (n=18)	PTZ-4 (n=18)	PTZ-7 (n=14)	FSE (n=10)
Time of Latency	55.6 $\pm$ 6.55	77 $\pm$ 28.95	93.80 $\pm$ 34.0	79 $\pm$ 31.0
Time of Seizures Grade I-II	263 $\pm$ 89.60	637 $\pm$ 162.30	429.20 $\pm$ 101.1 (n=12)	—
Time of Seizures Grade III-IV	—	—	1397.0 $\pm$ 164.8 (n= 2)	1442.1 $\pm$ 263.41 (n=10)

After 4 or 7 days of treatment, 4 rats with seizures Grade I-II were sacrificed in each time (PTZ-4, n=4; PTZ-7, n=4) respectively. Rats from PTZ4 or PTZ-7 groups were sacrificed one day after the last injection. The remaining ten rats were daily administered with PTZ up to present FSE and surgically treated after death, for heart removal.

As control for PTZ-4 and PTZ-7 groups, rats were daily injected i.p. with saline (1 ml/kg, i.p.) and then sacrificed after 4 (n=2) and 7 administrations (n=2). Two additional rats were sacrificed at day 9<sup>th</sup> and used as control of the FSE group. Animals were anaesthetized and fixed by perfusion for the immunohistochemical studies as indicated later.

The animal's care for this experimental protocol was in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience.

*b. Tissue preparation:* All brains and hearts were surgically removed and were placed in methyl-butane and frozen in dry ice. Frozen sections of 12  $\mu$ m were cut in a cryostat, thaw-mounted on gelatin-coated slides, and stored at  $-70^{\circ}\text{C}$  until the day of incubation.

*c. Immunostaining:* Tissue sections of control and experimental groups were simultaneously processed by immunofluorescent method. Initially, they were washed with PBS-Triton (PBS buffer containing 0.025% Triton X-100) for 15 minutes. Then, they were blocked during 1 h with normal horse serum (1:200) in buffer PBS. Antibodies were dissolved in PBS containing 1% v/v normal horse serum and 0.3% v/v Triton X-100, pH 7.4. The sections were initially incubated with anti-P-gp primary antibody (C-494 at a final concentration of 1:500) for 48 h at 4  $^{\circ}\text{C}$ , and then with fluorescent antibody anti-mouse conjugated (1:200) for 3 h (32). The contrasts were made with propidium iodure. Controls for the immunostaining procedure, routinely performed by omitting the primary antibody, did not develop any labeling. Anti-P-gp monoclonal antibody clone C494 was

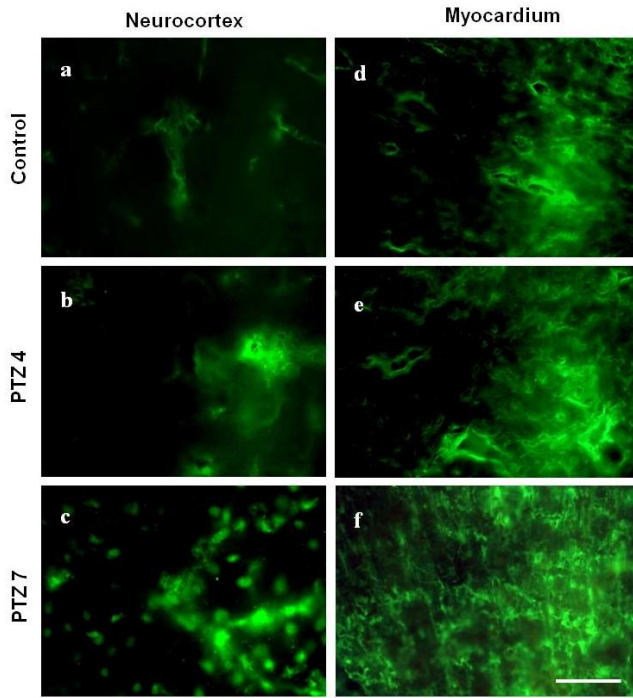
obtained from (Signet Laboratories, Dedham, MA) and fluorescent antibody anti-mouse IFTC conjugated was obtained (Zymed Lab Inc. San Fransisco, USA). Axio-photmicroscope and fluorescent pictures were taken in an Olympus BX-50 microscope equipped with a digital cooled camera (Coolpix). Figures were made with Adobe Photoshop 7.0 software.

*d- Statistics:* Individual experiments were composed of 6–10 tissue sections of each animal from each group. In all tissue sections quantification of P-gp immunostaining were recorded by arbitrary units of optical density and % of surface market, and differences among the means were analyzed using one-way ANOVA and Student–Newman–Keuls post test. Statistical significance was set to  $p < 0.05$ . Values represent the means of 3–4 independent experiments.

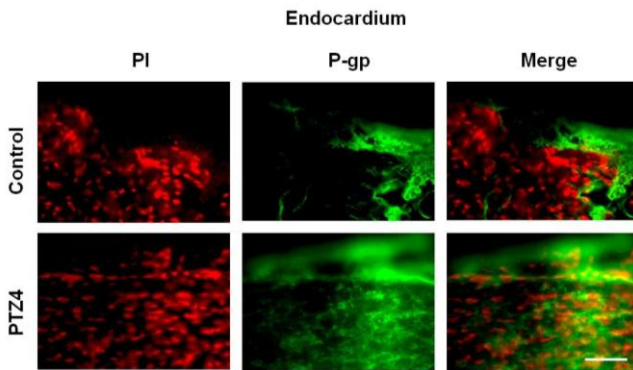
## Results and Discussion

All animals treated with PTZ developed seizures according with the severity scale used. Briefly, rats from PTZ-4 developed seizures grade I-II, while animals from PTZ-7 developed seizures grade I-II (n=12) or grade III (n=2), and remained alive. All animals with longer time of PTZ administration (n=10) developed seizures grade IV at 9<sup>th</sup> day, when died during SE. No significant differences in latencies were observed and the duration of seizures. However, a tendency to higher severity of scale used was observed from PTZ-7 group in 2 rats and in all rats at day 9<sup>th</sup> (Table 1).

In both brain and heart (pericardium, myocardium and endocardium) of control group, a basal expression of P-gp was detected, however a progressive increasing of this expression was observed in both tissues of all animals according with the number of days treated with PTZ (Figure 1). A details of this increased immunostaining is observed in pericardium at day 4 (Figure 2), and in myocardium and endocardium at days 4 and 7 (Figures 3 and 4). The effect was more evident in PTZ-7 group than in PTZ-4, when compared with control as evidenced by quantification of P-gp immunostaining in myocardium of



**Figure 1.** Progressive P-gp expression in neocortex and myocardium after repetitive seizures. Scale bar, 30  $\mu$ m.

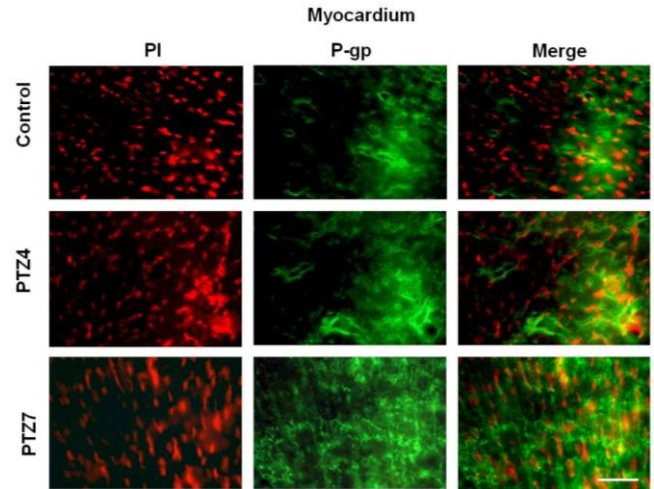


**Figure 2.** P-gp expression in endocardium in control and PTZ4 groups. (PI: propidium iodine). Scale bar, 30  $\mu$ m.

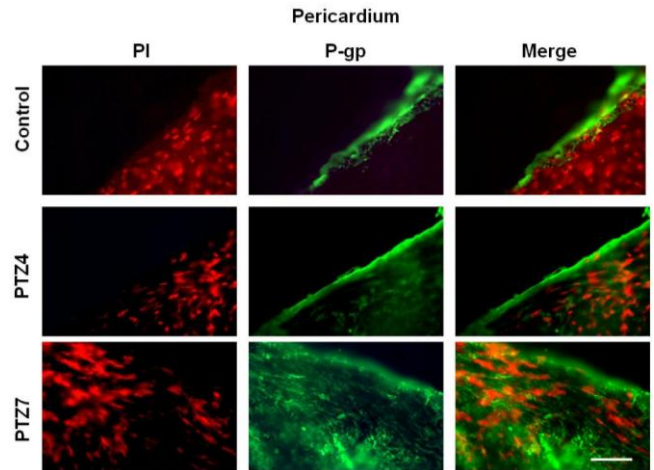
control and PTZ treated rats (Figure 5). In FSE group, a similar pattern expression of P-gp in myocardium as in the PTZ-7 group was observed (Figure 7). Coincidentally, a high increase of % of surface label in myocardium of both, PTZ-7 and FSE groups (Figures 5 and 8).

Pattern expression of P-gp immunostaining in brain (neocortex) of these animals (Figure 1) were similar to previously reported [27], and here we also show that the % of surface label in brain was PTZ-7>PTZ-4>controls (Figure 6), and this pattern was similar to the detected in myocardium (Figure 5).

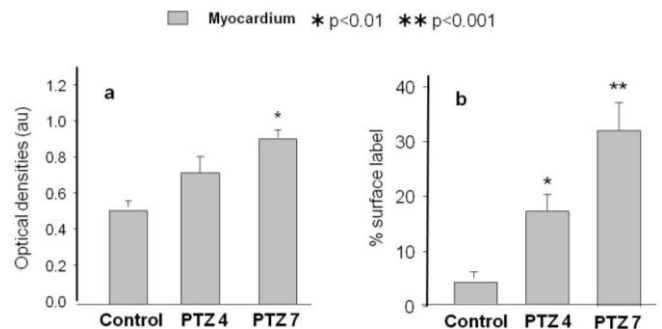
In our study, three main observations are remarkable related with repetitive convulsive seizures induced by



**Figure 3.** P-gp expression in myocardium in Control, PTZ4 and PTZ7 groups. (PI: propidium iodine). Scale bar, 30  $\mu$ m.



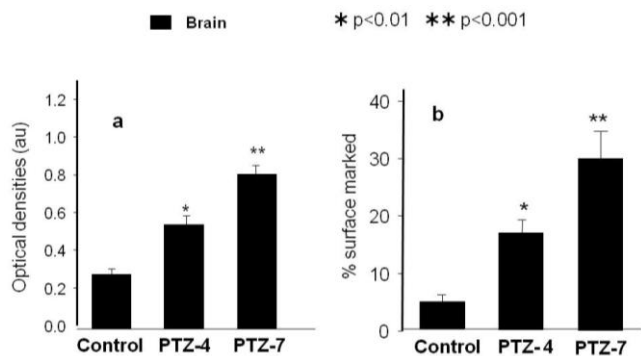
**Figure 4.** P-gp expression in pericardium in Control, PTZ4 and PTZ7 groups. (PI: propidium iodine). Scale bar, 30  $\mu$ m.



**Figure 5.** Image quantification of P-gp immunostaining in myocardium of control, PTZ-4 and PTZ-7 groups. **a:** Optical density; **b:** surface label (%).

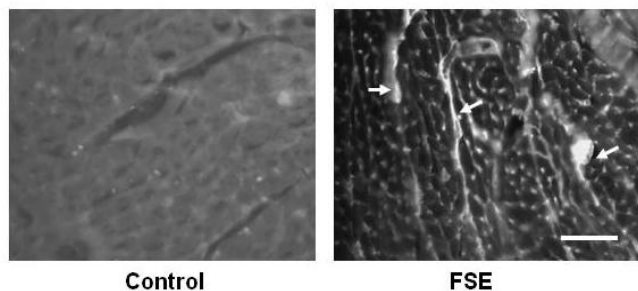
PTZ.

a: The progressive worsening in the severity of crises leading to FSE at 9<sup>th</sup> day.

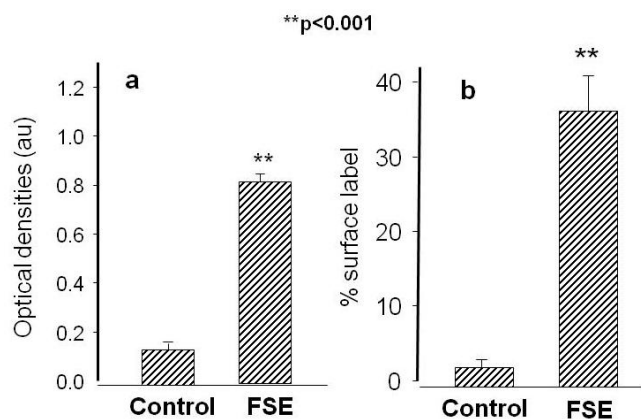


**Figure 6.** Image quantification of P-gp immunostaining in hippocampus of control, PTZ-4 and PTZ-7 groups. **a**-Optical density; **b**-surface label (%). Results corresponding to unpublished data from an additional study experimental repetitive seizure induced after daily consecutive doses of PTZ (see ref: 28).

**P-gp in myocardium**



**Figure 7.** Arrows indicate high P-gp immunostaining in myocardium FSE as compared with negative immunostaining in control. Scale bar, 30 µm.



**Figure 8.** Image quantification of P-gp immunostaining in myocardium of control and FSE. **a**-Optical density; **b**-surface label (%).

b: The simultaneous and progressive induction of P-gp expression in cells of brain and heart.

c: FSE was associated with higher P-gp expression in heart observed in all animals at 9<sup>th</sup> day.

The first concept is in agreement with previous results reported from our and other groups suggesting that progressive increased brain expression of P-gp can be induced by convulsive stress [16,29-32], where repetitive seizures, recruit more P-gp-positive neurons, inducing a progressive depolarization and encouraging the development of increasingly severe crisis [27], and triggering a FSE [19].

In the second point, it is important to highlight that the in normal heart, P-gp is absent or it is only expressed in endothelial cells of capillaries and arterioles, but not in cardiomyocytes [33,34]. Furthermore, P-gp overexpression in cardiomyocytes has been reported after both chronic and acute hypoxia-ischemic, and related with heart stunning [35,36].

At date, there are no reports showing cardiac P-gp overexpression after convulsive stress. So, our current data represent the first evidence showing a progressive expression of P-gp in heart secondary to repetitive seizures, an effect that appears to be increased according with the number and/or severity underwent seizures. Interestingly the P-gp expression in heart observed under control situation was restricted to membranes of vascular cells of myocardium as described in normal humans [33].

However, after repetitive seizures, a progressive increased expressing of P-gp was evident in cells of neocortex, pericardium, myocardium and endocardium (Figures 1-4).

It was not surprising to see an increased expression of P-gp in brain cells upon to convulsive stress as previously described [16,29-32].

Nevertheless, and similarly to the previously demonstrated in brain [27], the news is the P-gp expression in heart was higher in samples from PTZ-7 group than PTZ-4 group, indicating a parallelism between brain and heart of the progressive growing P-gp expression. Together, all these results suggest that during epilepsy, systemic factors involved in the up-regulation of *MDR-1* gen in heart as well as other peripheral associated organs could be related with the severity of the epilepsy. Furthermore, in an elegant experiment related with an altered phenytoin (PHT) pharmacokinetics, was documented the simultaneous high P-gp expression in different brain regions and liver of chronic epileptic rats [37]. In this mentioned article and in the current experiment, a common finding of high P-gp expression in peripheral organs (liver and heart respectively) was detected after persistent and repetitive convulsive stress.

Concerning the potentials mechanisms underlying the P-gp overexpression in heart, it was documented that convulsive stress, particularly tonic-clonic seizures and

prolonged tonic seizures can induce apnea and cyanosis, two well known hypoxic conditions<sup>[38,39]</sup>.

The continuous convulsive stress could mimic a repetitive apneas or hypoxia and produce heart ischemia. The MDR-1 gene is a survival rescue gene that can be up-regulated by several transcription factors including the Hypoxia Inducible Factor-1 $\alpha$  (HIF-1 $\alpha$ )<sup>[40]</sup>, the paradigm of hypoxia responsive elements<sup>[41]</sup>.

According with this mechanism, it was previously demonstrated that high P-gp expression in cardiomyocytes is observed after chronic heart ischemic insults, as documented by <sup>99m</sup>Tc-MIBI-SPECT<sup>[35]</sup>. Additionally, in an acute ischemic-reperfusion model, adult conscious sheep that underwent 12-min occlusion of the mid-left anterior descending artery followed by reperfusion, heart stunning was documented and high P-gp expression in cardiomyocytes was also observed, as soon as 3 hours after experimental artery occlusion-reperfusion procedure<sup>[36]</sup>.

Ischemia–reperfusion injury is a condition increasingly observed in clinical practice, given the widespread use of primary and rescue coronary angioplasty. When reperfusion occurs early enough to prevent cell death, the resulting feature is myocardial stunning<sup>[42]</sup> a phenomenon characterized by delayed recovery of ventricular contractile function despite successful reperfusion<sup>[43-45]</sup>. Several mechanisms related with oxidative stress and perturbation of calcium homeostasis with intracellular free calcium overload described in heart stunning<sup>[42,43]</sup>, are also observed in brain secondary to glutamate-dependent neurotoxicity<sup>[46]</sup>. In spite it was suggested that cardiovascular effects are probably not the major cause of SUDEP<sup>[48]</sup>, cardiac arrhythmias and cardiovascular changes are well documented during seizures and may be involved in some cases of SUDEP<sup>[35]</sup>.

In this regards, it was reported the potential pathomechanisms of SUDEP comprise cardiac arrhythmia, due to electrolyte disturbances, arrhythmogenic drugs, or transmission of the epileptic activity via the autonomic nervous system to the heart, central or obstructive apnea, and myocardial ischemia<sup>[28]</sup>. Furthermore, a variety of seizure-related cardiac dysrhythmias captured on ECG, including lengthening of the QT interval, ST depression and T-wave inversion, ventricular fibrillation and asystole, bradyarrhythmias, as well as atrial fibrillation, atrial and ventricular premature depolarizations, and sinus and supraventricular tachycardias were documented. Additionally, seizure-related apnea and hypoxia may also play a central part in potentiating cardiac arrhythmias, with subsequent hypoxia acting as an additional contributing factor in cardio-respiratory

arrest. Moreover, recently it was demonstrated that acute cardiovascular changes and death were observed after kainic acid-induced limbic cortical seizures in rats and was also suggested that the possibility of a coexisting “mild” susceptibility to sudden cardiac death, could be independent of the epilepsy, but which could become symptomatic in the presence of uncontrolled seizures<sup>[47,48]</sup>. All these controversial data, indicates that more studied should be developed to better understanding of heart mechanisms involved in SUDEP or FSE. The biological role for P-gp over-expressed in heart, still remain to be investigated, and it could open a new paradigm on acute heart failure in FSE and/or SUDEP.

Because P-gp over-expression also induce changes plasma membrane electrical potential producing membrane depolarization<sup>[22,23]</sup> it should be addressed related with electric function of the tissue or specific cells as neurons or cardiomyocytes. In our experiment we did not explored the cardiologic function, but taking in account all above mentioned, we speculate that the accumulative convulsive stress after repetitive seizure (no longer than few minutes), could also induce heart stunning. Under this hypothesis, the simultaneous seizure-dependent P-gp up-regulation in brain and heart observed, suggest a progressive risk to develop SE as we previously reported<sup>[17]</sup> and also could induce deleterious functional effects on heart. It could like a putative “mild” risk factor to develop cardiac failure as proposed in SUDEP and also under severe stress as occurring in SE. According with these observations, the mechanisms of sudden cardiac death in subjects with apparently normal hearts are poorly understood, however, seizure disorders were identified as co-morbid condition<sup>[49]</sup>. In this regards, it was demonstrated that chronic supplementation with omega-3 fatty acid restored the heart rate of rats with epilepsy toward control values and suggest a potential preventive effect of omega-3 FA supplementation against SUDEP<sup>[50]</sup>. Interestingly, a more recently published study demonstrated that omega-3 FA supplementation reduced the amount of P-gp contained in multidrug-resistant cancer cells, decreased the transporter activity, and restored the antitumor effects of different chemotherapeutic drugs<sup>[51]</sup>.

In summary, our results are the first evidence indicating that P-gp can be induced to overexpress in heart secondary to repetitive seizures.

Additionally, we can speculate that high P-gp expression in brain<sup>[17,27]</sup> could play a role on the risk for SE development, and high P-gp expression in heart as demonstrated here, could play a role in the risk for develop an acute and fatal heart failure.

Taking all data together, we believe that repetitive

seizures induce simultaneous P-gp overexpression in both brain and heart. It could be a combined risk factor to develop an acute heart failure under severe stress triggered by SE resulting in a fatal final as FSE. Because our experiments are only a descriptive study, where mechanistic and functional studies on heart were not developed, this hypothesis should be confirmed with further investigations.

### Conflict of interest

The authors confirm that this article content has no financial or non-financial conflicts of interest.

### Acknowledgement

This study was supported in part by the National Council for Sciences and Technology of Mexico (grant 98386), UBACyT M031 of the Universidad de Buenos Aires, Red GENIAR from Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo (CYTED), CECYT-CONACyT ME-PA03-SV-027 and Instituto de Ciencia y Tecnología del Distrito Federal (PFTU-08027).

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**To cite this article:** Auzmendi J, et al. Progressive heart P-glycoprotein (P-gp) overexpression after experimental repetitive seizures (ERS) associated with fatal status epilepticus (FSE). Is it related with SUDEP? *Mol Cell Epilepsy* 2014; 1: 43-51. doi: 10.14800/mce.66.