

# Lutein for alleviating early high mortality and brain pathology after experimental stroke in a genetic type I diabetic mouse model: abridged secondary publication

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## KEY MESSAGES

1. Hyperglycaemia plays an important role in the rapid exacerbation of stroke by compromising blood vessel integrity and increasing haemorrhagic transformation, resulting in extensive inflammation and high mortality.
2. These exacerbations are partially contributed by VEGF up-regulation, which has deleterious effects via triggering robust inflammation and vascular hyperpermeability.
3. After 0.5 hour of ischaemia, *Ins2<sup>Akita/+</sup>* mice displayed a delayed but significant development of infarct.
4. Lutein treatment reduced neurological deficits (after 0.5 hour of ischaemia) and mortality rate (after 2 hours of ischaemia).
5. Lutein is a potential treatment for stroke patients with type 1 diabetes.

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## Introduction

The incidence of stroke in patients with type 1 diabetes is four-fold higher than that in the general population. These patients are prone to die from stroke,<sup>1</sup> with shortened median survival<sup>2</sup> and more haemorrhagic transformation. We aimed to identify the causation of exacerbation of symptoms in patients with diabetes upon stroke using *Ins2<sup>Akita/+</sup>* mice, a model of type 1 diabetes carrying a point mutation in *Insulin 2 (Ins2)* gene.<sup>3</sup> Lutein is an anti-inflammatory and anti-oxidative agent that exerts neuroprotective effects in wildtype mice upon middle cerebral artery occlusion.<sup>4</sup> We aimed to determine the efficacy of lutein under hyperglycaemic conditions.

## Methods

Male *Ins2<sup>Akita/+</sup>* mice (11-16 weeks old) were used and kept under a 12-hour light/dark cycle with free food and water access. Experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong.

The mice were subjected to transient focal cerebral ischaemia using the intraluminal method.<sup>4</sup> Animals in sham control groups received the same experimental procedures except for filament insertion. Two hours of ischaemia and the corresponding sham operation were induced in both *Ins2<sup>+/+</sup>* and *Ins2<sup>Akita/+</sup>* mice, followed by either 2 or 22 hours of reperfusion (2hI/2hR and 2hI/22hR groups, respectively). 0.5 hour of ischaemia was induced only in *Ins2<sup>Akita/+</sup>* mice with either 3.5 or 23.5 hours of reperfusion (0.5hI/3.5R and

0.5hI/23.5hR groups, respectively).

In the 2hI/2hR group, lutein (0.2 mg/kg) was administrated intraperitoneally twice at 1 hour before and 1 hour after reperfusion. In the 0.5hI/23.5hR group, 2 mg/kg lutein was administrated once at 10 minutes before reperfusion. Control mice received the same treatment with 10% DMSO injection (vehicle).

The number of deaths was recorded, and neurological deficits were evaluated at the end of reperfusion using a scoring system.<sup>4</sup>

Brains were isolated and cut into five 2-mm-thick coronal slices using a brain matrix (RBM-2000C, ASI instruments). Brains slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 7.5 minutes and fixed in 10% buffered formalin overnight. The red and white regions (live and infarct tissues, respectively) on the posterior side were photographed and measured using Sigma ScanPro. The infarct areas and volume were estimated using an indirect method.<sup>4</sup> Haemorrhage transformations, as measured by total dark brown areas on the posterior side of TTC-stained brain slice no. 3 (approximately at bregma -0.34 mm), was presented as percentages.

For histological and immunohistochemical analysis, fixed brain slices were paraffin-embedded and sectioned (7 µm). Patches of orange red area after haematoxylin and eosin staining indicated the presence of haemorrhage. The sections were also immune-stained with anti-ZO-1 and anti-MMP-9 antibodies.

For Western blot analysis, brain lysates

were mixed with 2× ice-cold RIPA lysis buffer and analysed using antibodies against  $\beta$ -tubulin,  $\alpha$ -actin, matrix metalloproteinase (MMP)-2, MMP-9, ZO-1, vascular endothelial growth factor (VEGF), extracellular signal-regulated kinase (total Erk), p-Erk, p38 mitogen-activated protein kinase (total p-38 MAPK), and p-p38 MAPK. Signals were detected by ECL and quantified using Image J.

For real-time PCR analysis, brain lysates were mixed with ice-cold RNeasy lysis reagent and total RNA was extracted. cDNA was prepared from 2  $\mu$ g of extracted RNA. Real-time PCR reactions were performed based on SYBR Green technology using StepOnePlus system for:  $\alpha$ -actin, ATF6, BiP, CHOP, pERK, IRE1 $\alpha$ , Atg12, Bcl1, LC3-a, LC3-b, and p62.

Data were expressed as mean  $\pm$  standard error of mean or standard error. Survival rate and neurological score were analysed using the log-rank (Mantel-Cox) test and Mann-Whitney *U* test, respectively. All other measurements were analysed using one-way ANOVA, followed by Bonferroni post hoc test or unpaired student's *t*-test. A *P* value of <0.05 was considered statistically significant.

## Results

Ins2<sup>Akita/+</sup> mice showed decreased survival rate, worsen neurological outcomes, accelerated development of infarct, increased haemorrhage, and decreased vessel integrity. Ins2<sup>Akita/+</sup> mice showed significantly lower survival rate at 2 and 22 hours after reperfusion (Figs. 1a & 1b). Most deaths occurred in the first 4 hours after reperfusion in Ins2<sup>Akita/+</sup> mice but occurred generally later in Ins2<sup>+/+</sup> mice. Moreover, Ins2<sup>Akita/+</sup> mice had a more severe neurological deficit at 2 hours after reperfusion, compared with Ins2<sup>+/+</sup> mice (Figs. 1c & 1d). At 2 hours after reperfusion, infarct area was significantly larger in brain slice no. 1-3 of Ins2<sup>Akita/+</sup> brains, compared with Ins2<sup>+/+</sup> brains. The infarct area robustly exacerbated at 22 hours after reperfusion in Ins2<sup>Akita/+</sup> brains, compared with Ins2<sup>+/+</sup> brains (Figs. 1e to 1k).

Western blot analysis at 2 hours after reperfusion revealed that ZO-1 level was significantly reduced, the expression of MMP-9 (a matrix metalloproteinase known to disrupt the blood brain barrier following stroke) was increased (but not significantly), and the expression of MMP-2 was significantly up-regulated in Ins2<sup>Akita/+</sup> mice, compared with Ins2<sup>+/+</sup> mice (Fig. 2a). At 2 hours after reperfusion, inflammatory markers VEGF, p-Erk1/2, and p-p38 MAPK were significantly over-expressed in Ins2<sup>Akita/+</sup> brains, compared with Ins2<sup>+/+</sup> brains (Fig. 2a). Similarly, at 22 hours after reperfusion, VEGF expression remained significantly increased only in Ins2<sup>Akita/+</sup> mice (Fig. 2b). Significant increase of p-Erk1/2 expression was found in both Ins2<sup>+/+</sup> and Ins2<sup>Akita/+</sup> brains, yet the level in Ins2<sup>Akita/+</sup> mice was significantly higher. Increased CHOP expression

was also more pronounced in Ins2<sup>Akita/+</sup> mice at 2 hours after reperfusion.

Compared with vehicle mice, in Ins2<sup>Akita/+</sup> mice administration of two low doses of lutein (0.2 mg/kg) yielded a lower mortality rate in the 2hI/2hR group and significantly reduced neurological deficits in the 0.5hI/23.5hR group that had the same infarct size but milder neurological deficits (Fig. 3).

## Discussion

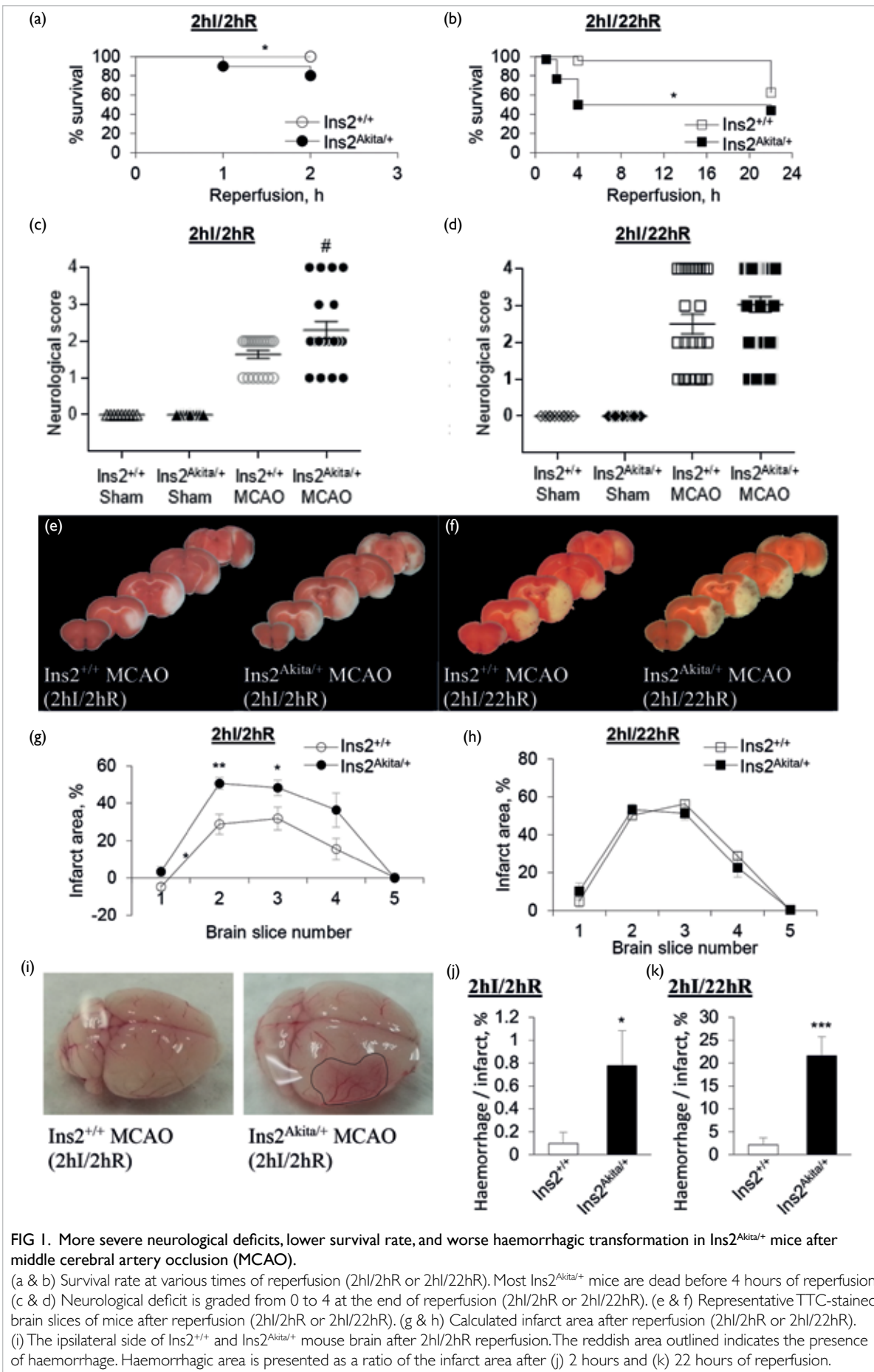
Induction of ischaemic stroke in hyperglycaemic Ins2<sup>Akita/+</sup> mice could mimic the exacerbated outcomes similar to those observed in patients with type 1 diabetes upon stroke. Earlier and more robust inflammatory responses and increase in pro-apoptotic CHOP were potentially responsible for the aggravation.

Haemorrhagic transformation was observed in Ins2<sup>Akita/+</sup> mice at 2 hours after reperfusion and robustly increased with reperfusion time. This was associated with heavier compromise of blood vessel integrity, which was proven in the greater loss of tight junction protein ZO-1 after 2 hours of ischaemia in Ins2<sup>Akita/+</sup> mice. These together with increased MMP-2 and MMP-9 expressions result in a more weakened blood vessel integrity and haemorrhagic transformation, which may account for further exacerbations and even the earlier death in Ins2<sup>Akita/+</sup> mice.

Besides the presence of haemorrhage, significant increase of inflammatory responses also worsened the outcomes in Ins2<sup>Akita/+</sup> mice. There was significant increase in p-Erk and p-p38 MAPK expressions only in Ins2<sup>Akita/+</sup>, and the increase persisted at high levels at 22 hours after reperfusion. This suggested long-term exposure of hyperglycaemia results in earlier and more robust Erk1/2 and p38 MARK activation, and thereby triggering inflammatory response and neuronal cell death.

The increase in CHOP expression shortly after 2 hours of reperfusion following 2 hours of ischaemia in both Ins2<sup>+/+</sup> and Ins2<sup>Akita/+</sup> mice may associate with apoptosis. The more profound up-regulation of CHOP in Ins2<sup>Akita/+</sup> mice may indicate additional apoptosis in the penumbra and infarct core under hyperglycaemia. CHOP up-regulation diminished at 22 hours after reperfusion, implying an early role of CHOP ischaemic stroke.

Most importantly, lutein treatment to Ins2<sup>Akita/+</sup> mice in the 2hI/2hR group resulted in a lower mortality. Moreover, in the 0.5hI/23.5hR group with milder neurological deficits, lutein was able to reduce neurological scores, suggesting a neuroprotective effect to the penumbra, likely via its anti-inflammatory and anti-oxidative property that counteracting damages from inflammation, free radical, and reactive oxidative species generated after ischaemia/reperfusion injury.



**FIG 1.** More severe neurological deficits, lower survival rate, and worse haemorrhagic transformation in Ins2<sup>Akita/+</sup> mice after middle cerebral artery occlusion (MCAO). (a & b) Survival rate at various times of reperfusion (2hI/2hR or 2hI/22hR). Most Ins2<sup>Akita/+</sup> mice are dead before 4 hours of reperfusion. (c & d) Neurological deficit is graded from 0 to 4 at the end of reperfusion (2hI/2hR or 2hI/22hR). (e & f) Representative TTC-stained brain slices of mice after reperfusion (2hI/2hR or 2hI/22hR). (g & h) Calculated infarct area after reperfusion (2hI/2hR or 2hI/22hR). (i) The ipsilateral side of Ins2<sup>+/+</sup> and Ins2<sup>Akita/+</sup> mouse brain after 2hI/2hR reperfusion. The reddish area outlined indicates the presence of haemorrhage. Haemorrhagic area is presented as a ratio of the infarct area after (j) 2 hours and (k) 22 hours of reperfusion.

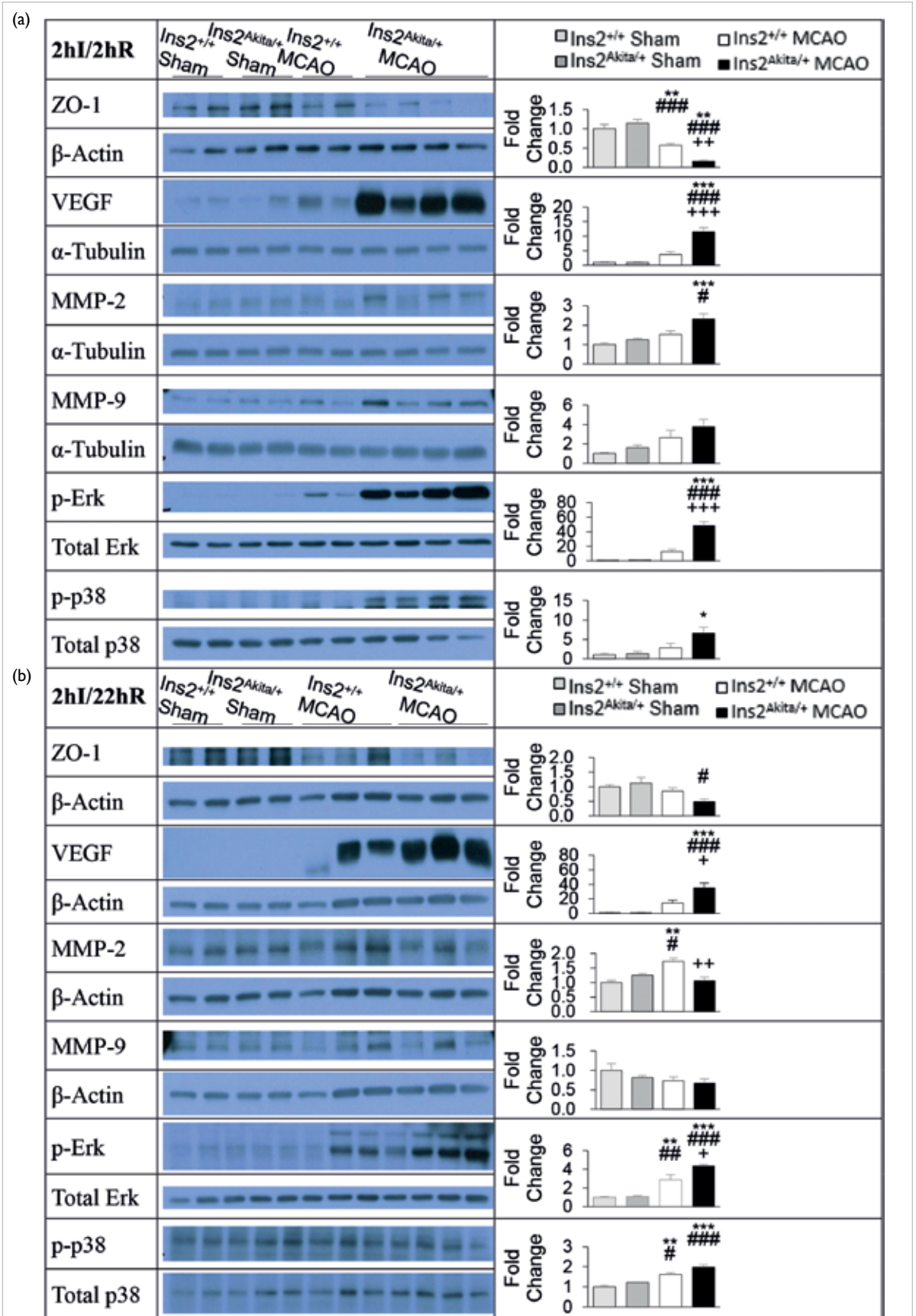
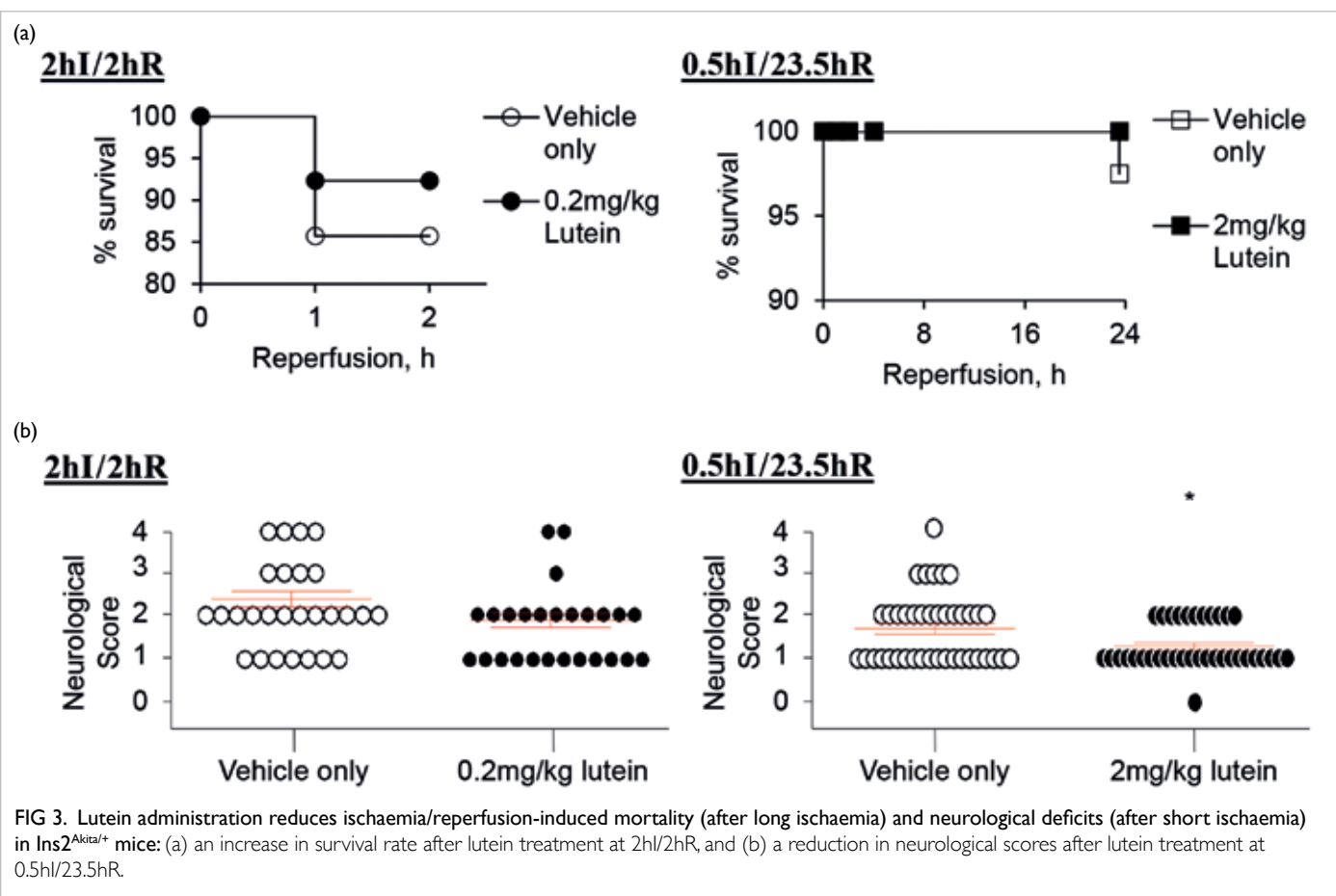


FIG 2. Vulnerable blood vessel integrity and increased inflammatory response in *Ins2<sup>Akit/+</sup>* mice at 2 hours after reperfusion: protein expressions of ZO-1 (a tight junction protein), VEGF, MMP-2, MMP-9, p-Erk, and p-p38 MAPK at (a) 2hI/2hR and (b) 2hI/22hR in *Ins2<sup>+/+</sup>* and *Ins2<sup>Akit/+</sup>* mice. Protein expressions are semi-quantified using Western blot analysis; corresponding fold changes are shown in the right panel.



## Conclusion

Hyperglycaemia plays an important role in the rapid exacerbation of stroke. After 2 hours of ischaemia, blood vessel integrity was compromised along with the presence of haemorrhagic transformation, extensive inflammation, and high mortality in diabetic mice at as early as 2 hours after reperfusion. We postulate these exacerbations are partially contributed by VEGF up-regulation, which has deleterious effects via triggering robust inflammation and vascular hyper-permeability. After 0.5 hour of ischaemia, hyperglycaemic *Ins2<sup>Akita/+</sup>* mice displayed a delayed yet still significant development of infarct. Most importantly, lutein treatment was able to lower mortality (after long ischaemia) and neurological deficits (after short ischaemia). Lutein is therefore a potential treatment for stroke patients with type 1 diabetes.

## Funding

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