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Characterization of a rat hypoxia-ischemia model where duration of hypoxia is determined by seizure activity

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ABSTRACT

Perinatal and early childhood asphyxia is common, debilitating and has few efficacious treatments. A hypoxia ischemia (HI) rat model that involves a unilateral ligation of the common carotid artery followed by a 60 min period of 8% oxygen hypoxia is often used to test proposed treatments. However, this HI protocol produces inconsistent infarction volumes due to the variability of individual rats to compensate for the ligated artery and hypoxia. Therefore, this HI model is problematic for experiments that prevent measurement of infarction volume, such as those that require analysis of homogenised brain tissue. We therefore aimed to find a simple and non-invasive predictor of infarction volume. Observations made prior, during and following HI in p26 rats showed that weight change 24 h following surgery was a strong predictor of infarction volume. The occurrence of a tonic clonic seizure during hypoxia was highly correlated with success of inducing an infarction, and for this reason we assessed whether ceasing the hypoxia for each rat following a tonic clonic seizure would produce a more consistent infarction volume. Using this procedure, infarction volumes measured at 3 and 15 days after surgery were significantly less variable, resulting in considerable improvements in statistical power compared with the original model.

1. Introduction

Perinatal and early childhood asphyxia is a major cause of morbidity and chronic cognitive dysfunction (Vannucci et al., 1999). In developed countries 1.1–14.3 births per 1000 have asphyxia related complications (Dzakpasu et al., 2009), including encephalopathy (Bass et al., 2004). Up to 50% of hypoxic ischemia induced encephalopathy cases lead to death, with a further 25% resulting in neurological impairment (Bass et al., 2004). At present, treatment is focused on managing the symptoms of perinatal asphyxia (Vannucci and Vannucci, 1997; Bhat et al., 2009), though recently, induced hypothermia has proven successful as a neuroprotective intervention (Hoehn et al., 2008).

For research into the development of neuroprotectants for perinatal asphyxia, the hypoxia-ischemia (HI) model developed by Rice et al. (1981) is the most widely used. This involves unilateral ligation of a common carotid artery in P7 rat pups, followed by a period of exposure to 8% oxygen/92% nitrogen mix in a temperature controlled chamber (hypoxia). This produces a large cerebral infarction that is similar in size and distribution to the adult rat transient middle cerebral artery occlusion model (Northington, 2006). By changing the length of hypoxia, this method has also been used successfully with rats and mice of varying ages (Gunn et al., 1990; Sheldon et al., 1998; Vannucci et al., 1999). One such variation involves the use of P26 rats with a 60 min hypoxia period (here termed "Fixed HI"). When comparing myelination, brain weight and neurogenesis this model is most analogous to early childhood asphyxia (Bayer et al., 1993; Rice and Barone, 2000).

Due to the variability in the physiological and anatomical capabilities of each rat to compensate for the occluded vessel, the infarction volume caused by Fixed HI varies greatly between rats, with some studies producing an infarction volume range of 0–82% of ipsilateral hemisphere (Brown, 1966; Saeed et al., 1993). As a result, experiments with Fixed HI have low statistical power. Furthermore, Fixed HI is problematic for assays that do not allow the measuring of infarction volume, such as those that require homogenised brain tissue (Saeed et al., 1993).

Because of these limitations we aimed to determine if any non-invasive observable factors assessed before, during or after HI could correlate with infarction volume, and evaluate if any of these potential correlates could be used as accurate predictors of infarction volume. Our results suggest that seizure-activity during HI is a more direct indicator of brain damage than the duration of hypoxia. We therefore designed a new variation on HI models, where hypoxia was terminated on the induction of a tonic clonic seizure (here termed "Variable HI"). In this paper

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we compare Variable HI with Fixed HI, and argue that Variable HI has several advantages with respect to testing neuroprotective agents.

2. Materials and methods

All procedures were carried out in accordance with the University of Otago Animal Ethics Committee guidelines on the care and use of laboratory animals. Unless stated otherwise, all chemicals used were acquired from Sigma–Aldrich, St. Louis, MO, USA, all surgical materials used were acquired from Southern Medical Products Ltd., Dunedin, New Zealand and all statistical analyses were performed on Minitab15® Statistical Software.

2.1. Hypoxia-ischemia surgery

A total of 48 male P26 Wistar rats (70–85 g) were anesthetized using 2.5% halothane in 100% oxygen. For the unilateral carotid artery ligation, a longitudinal incision was made immediately superior and lateral to the sternal notch and the left common carotid artery (CCA) was exposed by blunt dissection between the sternomastoid, sternohyoid and levator scapulae muscles. The vagus nerve and internal jugular artery were gently separated from the CCA, which was then permanently ligated using two 5-0 silk surgical sutures, and severed between the sutures. The incision was then closed with surgical clips, and animals were left to recover for a period of 2–3 h. After recovery, animals were put into an airtight chamber that was placed in a heated water bath. The floor of the chamber was insulated preventing direct heat transfer from the water through the chamber floor to the animals inside. The air temperature of the chamber was maintained at ±32.5°C and constant flow of a humidified (85–100% relative humidity) hypoxic gas of 8% oxygen/92% nitrogen was maintained through the chamber for 60 min in the first set of experiments, after which the rats were removed from the chamber. In a second set of experiments, each rat was removed from the hypoxia chamber immediately after a tonic clonic seizure occurred.

Immediately before, during and after HI the condition of each rat was closely monitored and several easily observable factors were measured. These factors included: body weight (g) measured every 24 h from 1 day prior to surgery to sacrifice, seizure events (as scored) during hypoxia, blood glucose prior to hypoxia (mmol), and rectal temperature prior to hypoxia (°C). Seizure activity was scored on a scale of 0–5 using the criteria detailed in Hesp et al. (2007). The highest seizure score that occurred for each 5 min within the hypoxic chamber was recorded. Total seizure score was calculated as the sum of the highest seizure score that occurred per 5 min over the 60 min. Maximum seizure score was calculated as the highest seizure rating observed during the 60 min.

Three days after HI, animals ($n = 18$) were sacrificed by decapitation and the brain rapidly removed and placed into an ice-chilled steel brain matrix. The matrix had gaps running transversely every 2 mm, in these gaps razor blades were placed slicing the brain into six 2 mm sections. The sections were then placed in a 12 well plate and washed with ice cold 20 mM phosphate buffered saline (PBS), before sections were incubated in 0.05% tetratrizolium chloride (TTC) (w/v) in 20 mM PBS (37 °C for 80 min in a darkened room). Sections were then washed with ice cold 20 mM PBS twice, and then incubated in 10% paraformaldehyde in PBS at 4 °C for 30 min (Joshi et al., 2004). Fixed sections were then photographed and the area of infarction was delineated manually using the Axiovision 3.1 software (Carl Zeiss). To correct for the distorting effects of brain swelling, we calculated infarctions by normalising to the contralateral hemisphere. This was done by calculating the infarction volume as the volume of contralateral hemisphere minus the volume of healthy tissue in the ipsilateral hemisphere, as described in Lin et al. (1993).

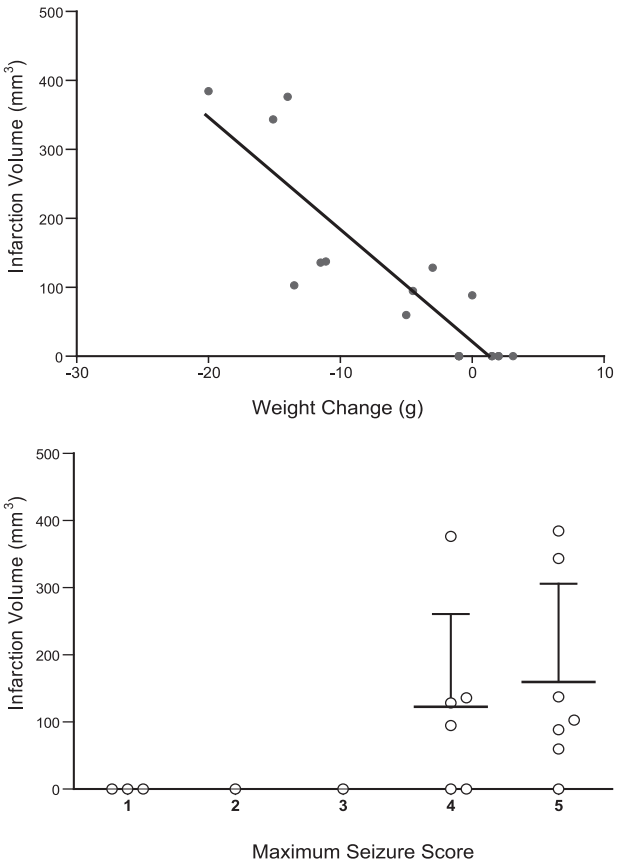

Fig. 1. (A) Weight change in the 24 h after HI compared with infarction volume measured 3 days after HI. The line was generated by linear regression. (B) Maximum seizure score and infarction volume measured 3 days after HI. A score of 5 represents a tonic clonic seizure. Horizontal lines represent a mean of the rats with that seizure score. Error bars represent SD.

Fifteen days after HI, the animals ($n = 16$) were sacrificed and the brains were quickly removed and placed in chilled 20 mM PBS. The brains were then embedded in ProLabo© optimum cutting temperature compound (VWR International) and frozen and stored at −18 °C. The embedded brains were then sectioned transversely using a cryotome. Due to the reduced size of the ipsilateral hemisphere, sectioning from the rostral end of the cortex would result in a misalignment of the sides of the brain in caudal sections. To reduce this effect, the striatum was used as the starting reference point, with the first section being 256 μm caudal to the rostral end of the both the ipsilateral and the contralateral striatums, and subsequent sections taken at 256 μm intervals. The sections were then stained using 0.1% (w/v) toludine/saline solution, washed in PBS and fixed using a 10% formalin solution. Each transverse section was then photographed using the Dinolite© camera (AnMo Electronics Corp., US) and the area of infarction was delineated manually using the Image J© software (National Institutes of Health, US). Infarction areas for each section were calculated as described for day 3 animals above.

2.2. Study design

A stepwise (forward and backward) iterative regression analysis was performed on all observed factors as predictors of infarction volume. The association of the occurrence of tonic clonic seizure and the occurrence of infarction was tested using a Chi squared test.

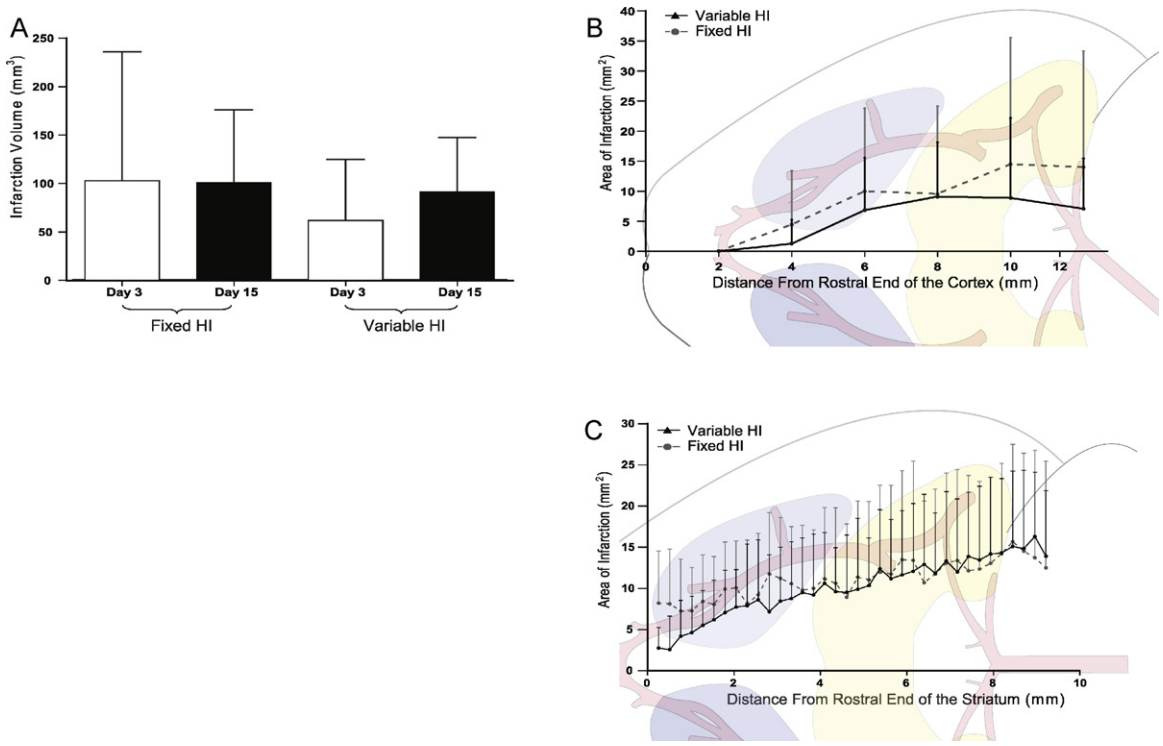

Fig. 2. (A) Mean and SD of infarction volumes for rats exposed to Fixed and Variable HI assessed at 3 or 15 days after HI. (B) Infarction areas for sequential sections in the ipsilateral hemisphere 3 days after HI for both Fixed HI ($n = 18$) and Variable HI ($n = 14$). The graph has been superimposed upon a diagram of the ipsilateral hemisphere, showing the position of the striatum (grey), hippocampus (light grey) and the main cerebral arteries. Individual points represent the means with error bars showing SD. (C) Infarction areas for sequential sections in the ipsilateral hemisphere 15 days after HI for both Fixed HI and Variable HI. Individual points represent a mean of eight rats and error bars represent SD.

After analysis of data from this initial experiment, we carried out a series of experiments comparing infarction volumes of Fixed HI with Variable HI. Infarction volumes for the two models were compared at 3 and 15 days after HI. Differences in infarction size between model protocols were assessed by two way ANOVA. Differences in the variance of infarction volumes between model protocols were assessed using the Bartlett's test.

Where possible all observations and measurements were made by an assessor blinded to the treatment group and to any previous measurements made on individual rats.

3. Results

3.1. Correlating factors of infarction volume in the Fixed HI model

Weight change during the 24 h after surgery, maximum seizure scores and total seizure scores during hypoxia were all significantly correlated with infarction volumes (Fig. 1A and B). Blood glucose levels and body temperature prior to hypoxia had no relationship with infarction volume. The stepwise multiple regression revealed that although maximum seizure score and total seizure did have a relationship with infarction volume, these factors co-varied with 24 h weight change, and 24 h weight change was a stronger predictor for infarction size ($R^2 = 0.77$). A Chi squared test revealed that there was a significant relationship between the occurrence of a tonic clonic seizure during hypoxia and the induction of an infarction ($P = 0.0011$). Because of the relationship between seizure activity and infarction volume, we then performed Variable HI on 22 rats, where exposure to hypoxia was terminated upon the occurrence of a tonic clonic seizure. Infarction volumes were measured at 3 days ($n = 14$) and 15 days ($n = 8$) after Variable HI. With Variable HI, there was no relationship with infarction volume and weight change nor between infarction volume and time in the hypoxia chamber (Fig. 3D).

3.2. Comparison of infarction size and distribution in Fixed and Variable HI

When the infarctions of both models were compared 3 days and 15 days after HI, we found that there was no significant difference in the size of the infarction (Fig. 2A). When the distributions of the infarction across the cortices were compared, it became evident that Variable HI produces less tissue loss in the rostral end of the striatum (Fig. 2B and C). It also appeared that at 3 days after HI, the Fixed HI produced more tissue loss in the hippocampus than Variable HI. However, this difference was not present at 15 days after HI.

3.3. Comparison of variability of infarction volume in Fixed and Variable HI

Infarction volumes were significantly less variable after Variable HI compared with Fixed HI at 3 days after HI (Fig. 3A) and moderately less variable at 15 days after HI (Fig. 3C). The reduced variation in infarction volume seen in Variable HI corresponds to a considerable increase in power, particularly when infarction volume was measured at day 3. Therefore, using Variable HI reduces the number of rats required to detect statistically significant reductions in infarction volume (Fig. 3B).

4. Discussion

4.1. Predictors of infarction size

Upon assessing the ability of several observable factors to be used as a predictor for infarction volume, we found that the most accurate was a linear regression of weight change in the 24 h following Fixed HI. All other factors co-varied with weight change,

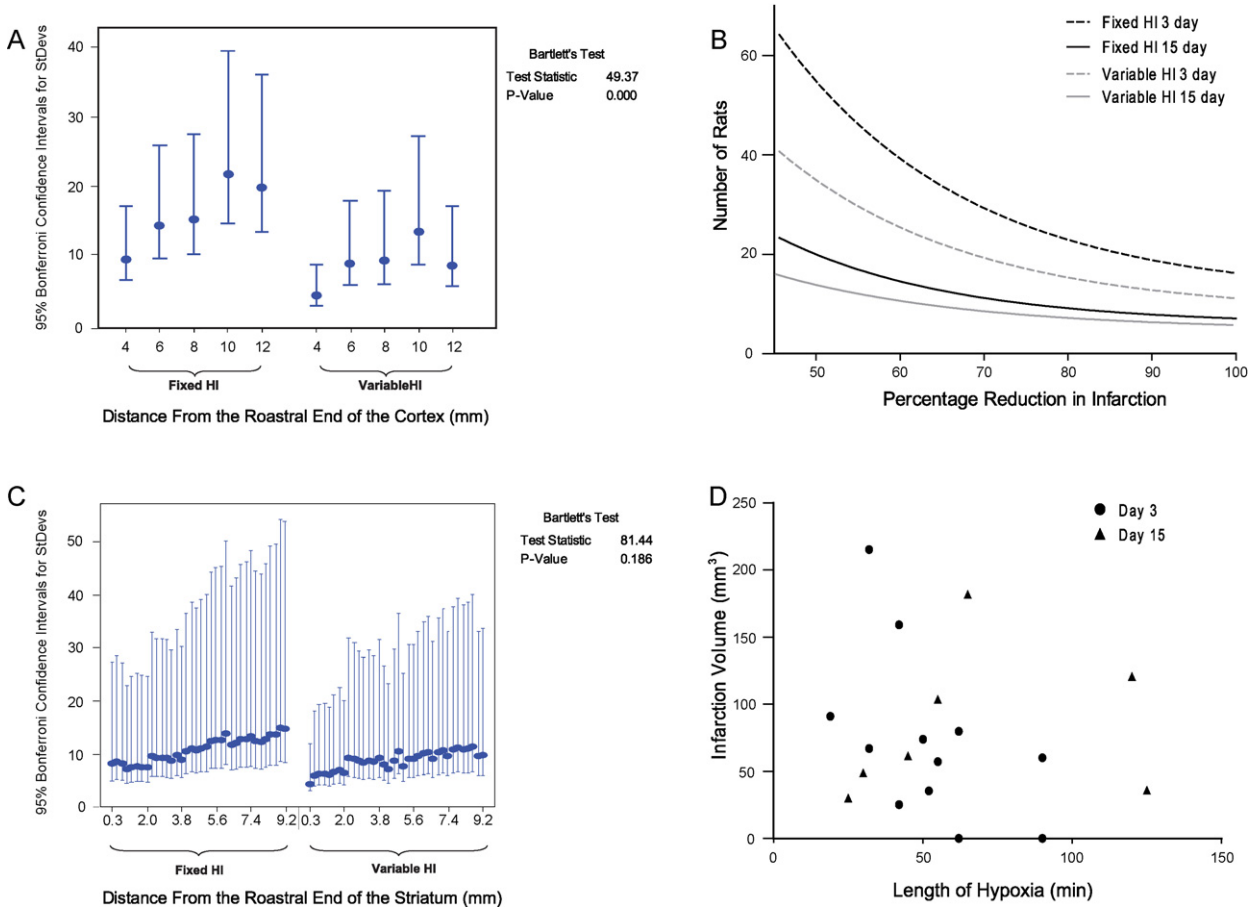

Fig. 3. (A) Median and 95% confidence intervals for standard deviations of infarction areas for sequential sections 3 days after Fixed HI or Variable HI. (B) Power analysis of Fixed and Variable HI, measured at 3 days and 15 days after HI, showing the number of rats required to assess a statistically significant difference, at differing levels of infarction volume reduction ($\alpha < 0.05$, $\beta < 0.2$). (C) Median and 95% confidence intervals for standard deviations of Infarction areas for sequential sections 15 days after Fixed HI or Variable HI. (D) Comparison of time spent in the hypoxic chamber before removal following a tonic clonic seizure and infarction volume in Variable HI (infarction volumes measured at 3 and 15 days after HI).

consequently a multiple regression could not be used to predict infarction volume. This relationship between weight change and infarction size is consistent with data described by Saeed et al. (1993), who reported a similar association between weight change over a 2 day period, in p7 rats, after HI. Weight change over the 24 h after HI is therefore a useful predictor of infarction volumes in experiments where it is not possible to measure infarction volumes directly, for instance experiments that require homogenisation of the brain for biochemical analyses. One complication of this relationship is that it may breakdown with experiments designed to test neuroprotective agents because energy expenditure and feeding behaviour could be affected by the neuroprotective agent, limiting the use of 24 h weight change as a predictor of infarction volume (Jensen et al., 1991; Croxford, 2003). The relationship between weight change and infarction volume was not present in Variable HI. We propose that this was due to the reduced variation of the model generating a tight cluster of both weight change and infarction volume, preventing the existence of a linear relationship between the two factors.

When assessing the correlation between maximum seizure score with infarction volume, we found there was a weak linear relationship (Fig. 1B). We then simplified the data into a binary form, the presence or the absence of an infarction and the occurrence of a tonic clonic seizure. In doing so, we found that there was a strong association between the occurrence of a tonic clonic seizure during hypoxia and the induction of an infarction measured 3 days after HI. Seizures alone do not result in neurological damage in neonatal animals (Wirrell et al., 2001). This suggested that tonic clonic seizures are a consequence rather than a cause of tissue damage during the hypoxia. This relationship led to the hypothesis that a higher infarction induction rate and a more consistent infarction would be seen in Variable HI, where hypoxia is terminated only after the occurrence of a tonic clonic seizure.

4.2. Comparison of Fixed HI with Variable HI

Our results show that Variable HI produces a more consistent infarction volume than the conventional Fixed HI model. Fixed HI assumes that equal duration of hypoxia gives a similar physiological challenge to individual animals. However, each individual rat varies with respect to their ability to compensate for a fixed duration of hypoxia. Variable HI is an attempt to control for this by assessment of an observable factor (tonic clonic seizure) that correlates with neurological damage, and ending hypoxia immediately after it occurs. Using Variable HI we found that the natural variability of Wistar rats to tolerate HI was very large, such that the time of hypoxia required to induce a tonic clonic seizure ranged from 19 to 125 min. Variable HI adjusts duration of hypoxia to the tolerance of individual rats to HI, as a result we found a reduction in variation compared to Fixed HI. This results in increased statistical power, lowering the number of rats required to test putative neuroprotectants. We propose that using Variable HI instead of Fixed HI would reduce both research costs and unnecessary animal suffering. However, it is possible that differences between

the two HI models change beyond 15 days after HI (Northington, 2006). Further studies would be required to test for this possibility.

4.3. Limitations of Variable HI

A limitation of Variable HI is that any drug administration must occur after the HI. Preadministration of a drug could change the seizure threshold causing early or later removal from the hypoxic chamber and result in a differing infarction to the vehicle group (Pisani et al., 1999). However, preadministration of drugs in animal models has been under recent criticism due to limited applicability to clinical practice (del Zoppo, 1998; Wang-Fischer, 2009; Rivers and Ashton, 2010).

As Variable HI requires constant observation and can last as long as 125 min, Variable HI is more labour intensive and requires more hypoxic gas than Fixed HI, but this is offset by the reduction in the number of animals required for the study.

5. Conclusion

Fixed HI produces a highly variable infarction and this makes it difficult to determine how changes in measurements taken from homogenised brain tissue are associated with the varying levels of brain damage. We found that 24 h weight change can be used to predict infarction volume, but that blood glucose and body temperature prior to Fixed HI has no effect on infarction volume within the range of natural variation. Seizure activity was found to be correlated with infarction volume in Fixed HI, as a result we designed a HI model that did not have a set time of hypoxia, and instead each rat was removed from the hypoxia chamber only after a tonic clonic seizure. As Variable HI produced a significant reduction in the variation of infarction volumes compared with Fixed HI, we propose that Variable HI represents an improvement on the original model.

Conflict of interest statement

We have no conflict of interests to declare.

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