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Recommendations for Preclinical Research in Hemorrhagic Transformation

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Abstract

Hemorrhagic transformation (HT) is an important complication of ischemic stroke and is responsible for most of the mortality associated with acute reperfusion therapy. Although many important publications address the preclinical models of ischemic stroke, there are no current recommendations on the conduct of research aimed at understanding the mechanisms and consequences of HT. The purpose of this review is to present the various models used in HT research, the clinical correlates, and the experimental variables known to influence the quantitation of HT in preclinical investigation. Lastly, recommendations for the conduct of preclinical research in HT are provided.

Keywords

hemorrhagic transformation; ischemic stroke; tissue plasminogen activator; animal models; hemorrhagic infarction

Introduction

Since long before the widespread use of intravenous tissue plasminogen activator (tPA) for the treatment of acute ischemic stroke, there has been great interest in studying the main adverse consequence of the therapy, hemorrhagic transformation (HT) [1]. HT is defined as hemorrhagic change in ischemic brain and includes a wide range of radiologic phenomena, from petechial hemorrhage to frank hematoma with mass effect [2]. Serious HT, leading to neurologic worsening, is quite uncommon in placebo-treated ischemic stroke patients (less than 1% within 36 hours in pivotal clinical trials) but those that receive tPA experience this at rates between 2.4% [3] and 6% [4]. This phenomenon has been widely accepted as an important barrier to the adoption of reperfusion therapy for stroke [5]. Preclinical research

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Conflict of interest

The authors declare they have no conflict of interest.

into the mechanisms and consequences of HT include animal models of nonhuman primate, cat, rabbit, rat and mouse [6–32]. The purpose of this paper is to review the occurrence and evaluation of HT in various animal models, identify clinical correlates and make recommendations for investigators to consider in performing preclinical HT research. The reader is referred to excellent published reviews for an exhaustive approach to all preclinical stroke models [33, 34].

Purpose of HT research

Preclinical investigation of HT can be performed for one of several purposes. By far the most common goal of HT research is to identify ways to ameliorate the adverse consequences of reperfusion therapy. In this context, HT research offers a venue to evaluate vasoprotective strategies and resultant therapies in stroke. Less common, but equally valid, is the study of the impact of vascular damage (as measured by HT) on stroke recovery and outcome. The choice of endpoints and models of HT may be quite different, depending on the goals of the research.

HT in animal models of stroke

Preclinical HT research includes a wide array of different species, from the nonhuman primate [6] to the transgenic mouse [29]. A list of the commonly employed animal models in HT research and the primary quantitation methods are given in Table 1. Increasing the duration of middle cerebral artery occlusion (MCAO) has been shown to increase the incidence [18, 19, 22] and consequence [18, 19] of HT, such that reperfusion with tPA before 3 hours is benign and after 6 hours is associated with a 50% incidence of parenchymal hematomas (PH) [18]. Although HT does occur in animals that are subjected to mechanical reperfusion of the MCAO [19, 21], and after permanent MCAO [31, 35], the size of the hemorrhage is increased almost 2-fold by the presence of tPA [12, 25].

Confounding factors/Important controls

An important factor to be considered in HT, and stroke research in general, is the inclusion of major risk factors that are present in a large proportion of patients with ischemic stroke. Elevated blood pressure [16, 36, 37] at the time of reperfusion and preexisting hypertension [38, 39] have been shown to consistently increase the likelihood of developing HT. Hypertensive animals have been shown to more reliably develop HT, especially when tPA is used in an embolic model and reperfusion is delayed until 6 hours after the onset of MCAO [12, 23].

In addition, hyperglycemia, either acute or due to diabetes, is also associated with increased incidence and severity of HT [20,40–42]. The incidence and severity of HT is much greater in diabetic animals, even in the absence of tPA [40–42].

It has also been shown that exposure to the inhaled anesthetic, isoflurane, increases HT [20]. In addition to all the important considerations in experimental stroke research in general [43], preclinical HT research should even more carefully control and report blood pressure and blood glucose. Rather a point estimate before, during and after MCAO, monitoring of blood pressure and blood glucose after administration of a test agent or during reperfusion and prior to sacrifice is recommended [16]. Blood pressure increases dramatically during MCAO in unanesthetized animals and remains elevated for days after the onset of reperfusion [44], pointing to the importance of monitoring in the reperfusion period. In addition, the total duration of anesthesia should be minimized and reported in order to interpret the results of intervention and improve translatability to humans, since they almost never receive anesthesia in the acute stroke period.

Evaluation of HT

Early investigations of HT in cats [8,9,45] identified the close relationship between the development of HT and reperfusion and subsequent mortality. Using visual inspection by a neuropathologist, the frequency and mechanisms of HT were clearly described using a dichotomized endpoint (presence or absence). It was in the earliest of these investigations [8] that the importance of reperfusion to the development of HT was reported. In cats exposed to either 6 or 24 hours of temporary MCAO, hemorrhagic infarction (HI) occurred in 40% and 60% of cases, respectively, when examined from 1–8 days after reperfusion. No HI occurred in permanent MCAO or in 1 hour temporary MCAO [8]. An attempt to quantify the degree of HT using a hemorrhage score was introduced in the rabbit [10], followed by the rat [19, 46–48] and mouse [28] models. Details regarding technical aspects of the rabbit embolic stroke model have recently been published [49]. Lastly, in search of a continuous endpoint for statistical comparison, many investigators have adopted a quantification of tissue hemoglobin, using a validated assay, as the primary endpoint of their HT research [16, 17, 20, 25, 26, 50]. To enhance clinical relevance, many investigators have also attempted to use categorical variables, either alone or in combination with one of the other parameters listed above [13, 14, 19, 23, 24, 31, 32] to describe the incidence and severity of HT development in preclinical models.

An important experimental caveat in the quantitation of HT is the limitation of the colorimetric hemoglobin assay. This assay is widely used for intervention studies but is subject to interference from interventions that stain the brain tissue. Agents such as minocycline and curcumin, have intense yellow coloring that can be detected in the visible light spectrum of the spectrophotometer (unpublished observations). When such agents are employed, they can cause false results in several ways. First, they can cause high absorbance yielding artifactually high hemoglobin values if background noise from the drugs is not considered. Second, the common practice of reporting excess hemoglobin in the ischemic hemisphere by subtracting the contralesional hemispheric value could lead to a falsely low hemoglobin value in the treated animals, complicating interpretation. Given these technical limitations, alternate methods of quantification of HT, such as visual observation and image analysis, should be employed to complement the results of hemoglobin assay [19].

Timing of Evaluation of HT

Unlike other experimental stroke models, where the timing of endpoint evaluation can vary from days to months after the onset of injury, most preclinical investigations of HT use a 24 or 48 hour time point (Table 1). Using repeated magnetic resonance imaging (MRI) assessment of rats subjected to 30 minutes of MCAO, it was demonstrated that all (100%) hypertensive animals develop some degree of HT by 7 days after the onset of MCAO, making a case for longer follow-ups in preclinical studies [23]. However, in this investigation, 23.1% of animals developed HT by 24 hours, with one-third being parenchymal hematomas (PH) [23]. In stroke patients, it is known that early HT, especially frank hematoma, is most often associated with clinical deterioration [51] and thrombolytic treatment increases HT within 36 hours of stroke onset [2]. Delaying the assessment of preclinical HT to beyond 3–4 days, where HT is present in more than 90% of animals [23] may increase the yield but lessen the sensitivity to detect the influence of intervention.

Clinical correlates

The classification of HT as either “symptomatic” (associated with neurologic worsening) or “asymptomatic” was used in the NINDS tPA Stroke Trial to determine the safety of tPA administered within 3 hours of the onset of ischemic stroke [4]. However, this classification has not been validated in animal models and the assessment of neurologic worsening is

confounded by the use of anesthesia and crude measures of neurologic function in animals. The use of the scoring system of the European Cooperative Acute Stroke Study (ECASS) investigators is entirely radiologic and has excellent reliability in detecting serious HT [2,52]. This imaging-based classification includes hemorrhagic infarction 1 and 2 (HI-1 and HI-2) and parenchymal hematoma 1 and 2 (PH-1 and PH-2) and has been shown to predict serious HT, leading to neurologic deterioration in rats as it does in human stroke victims [23]. These categories, in humans and in a preclinical model, are described in detail in Figure 1.

For HT investigations with the purpose of identification of interventions designed to reduce the consequences of thrombolytic therapy, the use of an embolic model (where a clot is placed endovascularly in a cerebral artery) has the most clinical relevance [53, 54]. Administration of tPA (or alternate thrombolytic agent) intravenously or intra-arterial [24] allows an important evaluation of the interaction with the test intervention [17, 26]. It is important to consider that vasoprotective agents that reduced HT when administered alone, have been shown to actually increase HT and worsen outcome when administered with tPA [26]. Another important aspect of HT research is that any intervention used in combination with tPA should be tested for its impact on thrombolysis [55]. An intervention that reduces the thrombolytic effect of tPA would also reduce HT, but with no clinical benefit.

Recent clinical evidence has emerged to support the notion that HT, even if initially asymptomatic, increases the risk of long-term disability [56]. However, experimental evidence on the impact of HT on functional recovery is scarce and merits further investigation. Preclinical investigations designed to determine the impact and mechanisms of vascular damage after cerebral ischemia employ models that may or may not include a thrombolytic agent [19].

Mortality

Serious HT has been shown to increase mortality in stroke patients (3,4) but mortality rates are not reported in all preclinical investigations of HT. Mortality in animal models of HT vary based on the duration of occlusion, the timing of administration of a thrombolytic and the survival time. In normotensive rats subjected to either 5 or 6 hours of embolic MCAO, followed by tPA, 24 hour mortality rates of 48 – 55% have been reported, compared to 14 – 18% in permanent MCAO [18, 57]. However, when tPA was administered within 3 hours, mortality was only 13% [18]. The presence of hypertension [23] or hyperglycemia [15, 20] increases the severity of HT at shorter durations of ischemia and increases mortality [15] at 24 hours. In order to compare the results of preclinical investigations from different laboratories, mortality rates should be reported.

Recommendations for preclinical research in HT

In summary, the following recommendations can be made:

1. Studies of HT development after reperfusion therapy should employ an embolic model and a translatable assessment of HT severity (categories).
2. Blood pressure and serum glucose should be monitored and reported before, during and after MCAO in addition to during reperfusion and prior to sacrifice.
3. Duration of anesthesia for each animal should be recorded and means compared between treatment groups.
4. Use multiple methods of quantitation: presence/absence; graded; HT area; hemoglobin assay.

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Figure 1.

Categories of hemorrhagic transformation in a preclinical rabbit embolic model (top) and human stroke victims (bottom). The top 4 images are examples of fresh brain slices from a rabbit large clot model, treated with tPA and sacrificed from 12–24 hours after stroke. The bottom 4 computed tomography (CT) images are from human stroke victims, approximately 24 hours after treatment with tPA for acute ischemic stroke. HI1 = hemorrhagic infarction 1: petechial hemorrhage; HI2 = hemorrhagic infarction 2: confluent petechial hemorrhage; PH1= parenchymal hematoma 1: hematoma less than 30% of the infarct with mild mass effect; PH2= parenchymal hematoma 2: hematoma greater than 30% of the infarct with significant mass effect.

Table 1

Hemorrhagic transformation in animal models.

Species	Clot	tPA	Quantitation Method	Survival time(s)	References
Nonhuman Primate	No	No	Presence/Absence	1 h-7 days	[6]
	No	No	Hb Assay (ELISA)	24 h	[7]
Cat	No	No	Presence/Absence	1-8 days	[8]
	No	No	Presence/Absence	2 weeks	[9]
Rabbit	Yes	Yes	HT Presence/Score/Categories (PH, HI)	48 h	[10]
	Yes	Yes	Presence/Absence	6 h	[11]
Rat	Yes	Yes	Hb Assay (colorimetric)	24 h	[12]
	No	No		24 h	[13]
	Yes	Yes		24 h	[14]
	No	No		24 h	[15]
	No	No		24 h	[16]
	Yes	Yes		24 h	[17]
	Yes	Yes		24 h	[18]
	No	No		24 h	[19]
	No	No		24 h	[20]
	No	No		24 h	[19]
Mouse	No	Yes	Presence/Absence	24h	[21]
	No	No	Area (image analysis)	24 h	[19]
	No	Yes	Area (image analysis)	24 h	[22]
	No	No	Categories (PH, HI)/score	1-7days	[23]
	Yes	Yes		24 h	[18]
	No	Yes	Hb Assay (colorimetric)	24 h	[24]
	Yes	Yes		24 h	[25]
	No	Yes		24 h	[26]
	Yes	Yes	Presence/Absence	24h	[27]
	No	No		24 h	[26]
	No	No		24 h	[28]

Species	Clot	tPA	Quantitation Method	Survival time(s)	References
	No	No	Area (image analysis)	24 h	[29]
	No	Yes		24 h	[30]
	No	No	Score	48 h	[31]
	Yes	Yes	Categories	24 h	[32]