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Review Article

Using hormetic strategies to improve ischemic preconditioning and postconditioning against stroke

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Abstract: Both ischemic preconditioning (IPreC) and ischemic postconditioning (IPostC) trigger endogenous neuroprotective mechanisms in cerebral ischemia. IPreC is defined as a brief ischemia that protects against a subsequent severe ischemia, while IPostC refers to a series of brief cerebral blood vessel occlusions performed at reperfusion following an ischemic event. Hormesis describes a biphasic dose-response relationship in toxicology, where a low dose of toxicant stimulates and a high dose inhibits biological responses. In general, any minor stress will stimulate a biological system to generate an adaptive response; in most cases, if not all, such an adaptive response to a minor stress is beneficial to the biological system. Proponents of hormesis suggest that this effect is independent of any models, either in vivo or in vitro, from animal, plant, fungi, yeast, to bacteria, by any measurement of end points, survival ratio or time, growth, tissue repair, life span, cognition, learning and memory. In this review, we examine whether IPreC and IPostC are actually sub-forms of hormesis and whether quantitative hormetic strategies can be used to study IPreC and IPostC. By integrating the concepts of IPreC and IPostC with hormesis, we aim to broaden the avenues leading to clinical translation of IPreC and IPostC in stroke treatment.

Keywords: Ischemic postconditioning, preconditioning, stroke, hormesis

Introduction

Stroke is a leading cause of death and significant long-term disability in adults worldwide. No effective treatment exist, except for the FDA approved-thrombolytic agent, t-PA, which can only be applied to a small population of stroke patients in a short therapeutic time window from 3 to 4.5 hours after stroke onset [1]. Numerous innovative neuroprotectants have been proposed for clinical translation to treat stroke in the last few decades, including Ca^{2+} antagonists [2, 3], glutamate inhibitors [4-6], free radical scavengers [7-9], necrotic and apoptotic blockers [10-13], anti-inflammatory agents [14-16], induced-mild to moderate hypothermia [17-19], and others. Due to the triggering of intrinsic protective mechanisms in the brain, the concepts of ischemic preconditioning (IPreC) and ischemic postconditioning (IPostC) are especially attractive as neuroprotectants involved in multiple cell signaling pathways [20, 21]. The protective effects of IPreC are considered the gold standard for stroke protection.

The primary goal of studying IPreC and IPostC against stroke is clinical translation to patients [22]. Even though none of the patterns of IPreC have yet been successfully translated to stroke patients after decades of research, our search continues. While the concept of IPostC is relatively new, the potential for clinical application remains unknown. Despite the paths carved out by the research in IPreC and IPostC, is the road ahead even more difficult leading to clinical application? Are the protective effects of IPreC and IPostC proven in the laboratory merely illusions in the clinical setting? Are there innovative concepts and strategies that can broaden the narrow path and make clinical translation of ischemic pre- and post-conditioning a reality? Mindful of these concerns, we intend to introduce the concept of hormesis in IPreC and IPostC against stroke, assuming that many researchers are unaware of this concept. Terms

such as preconditioning hormesis and postconditioning hormesis have been proposed to describe preconditioning and postconditioning [23-25], but these terms are not widely used in the stroke field for pre- and post-conditioning studies and leading stroke researchers have not yet accepted the concept of hormesis as different patterns of IPreC and IPostC. We intend to better define hormesis as it relates to IPreC and IPostC in our ongoing studies. By integrating the concept of IPreC and IPostC with hormesis, we aim to broaden the avenues leading to clinical translation of IPreC and IPostC in stroke treatment.

Both IPreC and IPostC protect against stroke

IPreC refers to a brief ischemia that protects against a subsequent severe, prolonged ischemia. This protective phenomenon can be traced back nearly 50 years, when a research group documented increased survival times in rats, with reductions in hippocampal CA1 neuronal loss during early exposure to brief anoxia [26, 27]. These findings were not recognized at the time as a type of IPreC. The formal concept of IPreC was first described in ischemic hearts by Murry et al. in 1986 [28]. It was later found that IPreC has two therapeutic time windows. Rapid IPreC is conducted within a few hours of the first ischemic event, and delayed IPreC is performed 24 hours or longer before the second, prolonged ischemia [29, 30]. Many independent studies have demonstrated the robust cardioprotective effect of ischemic myocardial preconditioning across several species tested with different protocols [31]. In addition to myocardial ischemia, the protective effect of IPreC has been reproduced in other organs including the liver [32], kidney [33] and brain [34].

The formal concept of cerebral IPreC was first introduced in the early 1990s by Kitagawa et al., in a global cerebral ischemic rat model [20, 35]. After that, many independent groups confirmed the protective effects of IPreC in both global and focal cerebral ischemia across different species both in vivo, including gerbils [36-38], rats [39-41], and mice [42], and in vitro experiments, including brain slices [43] and cell culture [44].

In contrast, the concept of IPostC is relatively new. IPostC was also initially defined in the field of myocardial ischemic research [45, 46]. While IPreC is a sublethal ischemia performed in advance of a severe ischemia, IPostC conventionally refers to a single brief or a series of brief occlusions/reperfusions performed after ischemia/reperfusion. The protective effect of IPostC has also been proven against cerebral ischemia [21, 47], and has been shown to be comparable to that of IPreC. The therapeutic time window of IPostC against cerebral ischemia can vary from a few seconds to a few days after ischemia/reperfusion. Taken together, IPostC and IPreC confer significant neuroprotective effects on brain ischemia.

The concepts of IPreC and IPostC have expanded to represent a broad range of sublethal insults, from ischemia, neurotoxic agents and pharmacological agents, to physical exercise [34, 48-50]. Recently, remote pre- and post-conditioning have received particular attention, especially in the myocardial ischemia research field, due to their relative safety for clinical translation [51-55]. Remote pre- or post-conditioning refers to an ischemia performed in a remote, uninvolved extremity, such as a leg or an arm, which generates protection against another ischemic event in a vital organ, such as the brain or heart [56-62]. Remote conditioning has been applied in many pilot clinical trials and has proven effective in myocardial ischemia, which may shed light on the potential clinical success of pre- and post-conditioning in stroke treatment [63-67].

The concept of hormesis

As reviewed previously [68], German pharmacologist Hugo Schulz first described such a phenomenon in 1880s after observing that the growth of yeast could be stimulated by small doses of poisons. The term “hormesis” was coined and used for the first time in a scientific paper by C.M. Southam and J. Ehrlich in 1943 [68]. Recently, Edward Calabrese has revived the hormesis theory through a series of his publications by examining hormetic phenomenon across multiple research disciplines of biological sciences [25, 68-71]. In toxicology, three models exist to address dose-dependent response relationships: a threshold model, a linear model and a hormetic model (**Figure 1**), which were well addressed by Dr. Hoffmann in his excellent review [72]. Briefly, in the threshold model, a toxicant has no effect below a threshold of dose response curve, but above

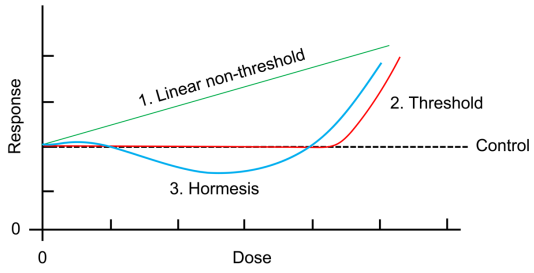

Figure 1. Three models for a dose-response relationship in toxicology (modified from [72]. 1. Linear non-threshold model; 2. Threshold model; 3. Hormetic model. The control line shows the baseline when a biological system is not challenged by a stress. The "J" or "U" shaped curve in 3 shows the hormetic dose-response of a toxic agent on a biological system or organism. Lower dose ranges of the toxic agent are beneficial while doses above the threshold are inhibitive or detrimental.

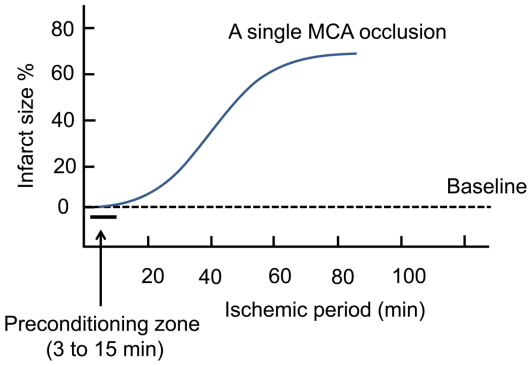

Figure 2. A single ischemia (MCA occlusion) as preconditioning does not generate a hormetic response, but instead a threshold curve. The baseline indicates no infarction.

the threshold the toxicant generates a dose-dependent toxic response. As a result, a higher dose corresponds to a stronger toxic effect. The linear model has no effect threshold. Instead, any toxicant dose generates a proportional dose-dependent effect, producing a descending line when doses are reduced until a zero effect is reached. Both dose-response relationships in the linear and threshold models are monotonic. In contrast, the hormetic model produces a biphasic dose-dependent response [69, 73]. The effect does not change in one direction in response to dose changes. Rather, lower doses generate opposite effects to higher doses. If higher doses of an agent produce detrimental effects to a biological system, lower doses produce beneficial effects and, conversely, if higher doses generate beneficial effects, lower doses generate detrimental effects. Hormesis can therefore be defined as a dose-response relationship in which high and low doses of an agent have opposite effects. In the field of toxicology research, hormetic dose-dependent effects have been shown for many chemicals [71, 74-77] as manifested by J- or U-shaped profiles [25, 72] (**Figure 1**).

Whether hormesis is a universal, default phenomenon for all toxicants is an issue of debate, but a strong proponent of hormesis, Dr. Edward J. Calabrese, considers it a universal phenomenon, not only in toxicology, but also in immunology, aging biology, psychology, neuroscience, ecology, plant biology, microbiology, radiology, and many other sub-disciplines of biological and medical science [25, 69, 71, 73-76].

Are IPreC and IPostC forms of hormesis?

As discussed, strong proponents of hormesis have suggested it is a universal phenomenon across the kingdom of biological sciences. However, hormesis is not commonly recognized, in part because different research fields use many alternate terms for it when studying dose-response relationships. These terms include non-monotonic, biphasic, U-shaped, J-shaped, rebound effect, bitonic, preconditioning, postconditioning, and adaptive response, among others [69, 71, 73]. Intended as an effort to promote communication among scientists from different fields, in 2007 Dr. Calabrese and nearly 60 other biomedical scientists advocated integrating biological stress responses in the hormetic context, including pre- and post-conditioning, and recommended a terminology system for each stress/hormesis based on an interdisciplinary framework [25]. The terms preconditioning hormesis and postconditioning hormesis were suggested to replace pre- and post-conditioning, respectively [25]. These terms, however, have not been officially adopted by researchers in the pre- and post-conditioning fields, including myocardial ischemia, cerebral ischemia, kidney or lung ischemia, even 5 years after publication of the paper. There are several explanations for this situation. Most of the proponents for the new terminology do not conduct related research, and many scientists who study pre- and post-conditioning are unaware of the hormesis concept and terminology system. The concepts and

definitions of pre- and post-conditioning may already be sufficient to researchers in the field, rendering new terms unnecessary. A careful and serious examination of whether pre- and post-conditioning truly fall within the hormetic context has not been experimentally tested nor analyzed.

In our opinion, the concepts of IPreC and IPostC may differ from classic hormesis in several aspects. First, classic hormesis refers to low doses of a toxicant generating beneficial or stimulatory effects to a biological system. End points include enhanced growth, survival and life span. These measurements of end points are independent, and do not account for any additional insult or stress to the observed biological system. Manifestation of the beneficial effects of IPreC and IPostC are dependent on another prolonged ischemic event, which occurs either after or before induction of IPreC or IPostC, respectively. In other words, without the prolonged ischemia as a reference, the beneficial effects of IPreC and IPostC cannot be demonstrated. Second, IPostC is less likely to be listed under the hormetic context than IPreC, as the protective mechanisms of IPostC are less well understood. The effects of IPreC alone on a biological system can be studied. It is generally accepted that IPreC causes an adaptive response in the targeted organ for the subsequent prolonged ischemia. Thus, IPreC is more like a hormetic agent, except the protective outcome of IPreC must be measured with a reference from the aforementioned subsequent prolonged ischemia. It seems impossible to separate an IPostC event from its preceding ischemia and use IPostC alone as a research subject. It seems meaningless to study how this IPostC event affects the brain without the accompanying prolonged ischemia. A series of very brief occlusions (from 10 to 30 seconds for a few times) alone may not significantly alter normal brain function, and investigation of this subject may not offer any meaningful clues for understanding the protective mechanisms of IPostC. Such forms of IPostC may not be "stresses" as defined for hormesis. Rather, IPostC attenuates a stress caused by the previous prolonged ischemia, as IPostC interrupts reperfusion and attenuates ROS production. If this is true, it is inappropriate to include IPostC in the context of hormesis. We believe, however, that whether IPostC generates an adaptive response or produces a compensatory effect similar to a hormetic agent requires more careful study.

IPreC alone shows features of the threshold but not the hormetic model when infarction is measured

We further examine and discuss whether IPreC alone generates a hormetic response in animal models of the preconditioned brain. IPreC is usually induced by a brief ischemia, which is arbitrarily defined in many studies as from 2 to 15 minutes of MCA occlusion [78-83]. It is known that ischemia causes ATP depletion resulting in ischemic or anoxic depolarization [84]. Previous studies have shown that the occurrence of ischemic depolarization is proportional to the degree of neuronal death [85, 86]. It usually requires more than 2 to 3 minutes under normothermia to cause ischemic depolarization [87-90]. Previous studies often defined IPreC as 3 to 15 minutes of MCA occlusion, which reduces infarction induced by a subsequent severe stroke [78-83, 91]. This raises the question as to whether IPreC with 3 to 15 minutes of MCA occlusion causes any neuronal injury or infarction to the brain. Although IPreC was originally defined as a sublethal ischemia, unfortunately in most studies, whether a brief ischemia causes brain injury or not was not carefully examined or reported. Under the threshold of less than 2 to 3 minutes of ischemia, no ischemic depolarization occurs, and no neuronal death or infarction will be induced, but it may also not produce protection as a preconditioning factor. From forebrain ischemia, it is known that only 3 to 5 minutes of ischemia is sufficient to induce delayed, selective neuronal death in the cerebral cortex, striatum and hippocampus [92, 93]. In focal ischemia, brain injury is often measured by TTC staining, which cannot measure neuronal injury induced by 3 to 5 minutes of ischemia with selective, delayed neuronal death rather than gross infarction. Therefore, to conclude that IPreC with 3 to 5 minutes of focal ischemia does not induce any neuronal death might be misleading and incorrect. When the ischemic period is increased above the threshold of ischemic depolarization, it is imaginable that neuronal death or infarct size will be increased along with the increases in ischemic period. Indeed, in our laboratory clear infarction can be detected after 10 to 15 minutes of MCA occlusion (unpublished observation). If brief ischemia

from 3 to 15 minutes is used as preconditioning period, minor brain injury is expected, at least in the ischemic core, where infarction is visible with 10 minutes of ischemia. With longer periods of ischemia, infarct sizes will be increased toward ischemic penumbra. Obviously, the dose-response relationship between ischemic time of preconditioning and infarct sizes will fall into the category of the threshold model (**Figure 2**), rather than a hormetic model. Therefore, it is safe to conclude that IPreC alone does not generate a hormetic response when infarction is used as a pathological measurement.

We recognize, however, that the above conclusion is based on infarction measurement, and hormetic response may be observed at cellular and molecular levels. For instance, Ca^{2+} is known to be critical for neuronal death after stroke. When IPreC preceded a lethal 5 minute forebrain ischemia in gerbils, enhanced plasma membrane Ca^{2+}-ATPase activity and increased mitochondrial sequestration Ca^{2+} was present before the subsequent test ischemia was induced [94]. IPreC also promotes hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates the adaptive response to hypoxia in mammalian cells [95-97]. It promotes protein expression of excitatory amino acid transporters, which helps inhibit glutamate-mediated synaptic signaling and attenuate extracellular glutamate levels preventing its neurotoxic activities [98]. Furthermore, protein expression and activities of anti-oxidant proteins, such as SODs *(*Mn-SOD and Cu/Zn-SOD*)*, glutathione peroxidase and catalase, are increased by IPreC [99]. IPreC also increases protein expression of heat shock proteins (HSPs) [99], a family of stress proteins that act as molecular chaperones, proven to be neuroprotective [16, 100]. Furthermore, IPreC promotes protein expression and activities in the Akt cell signaling survival pathway [101], and enhances anti-apoptotic protein levels of Bcl-2, Bcl-XL while inhibiting pro-apoptotic protein levels of Bax, Bad in the Bcl-2 family [99]. However, these results are often considered controversial in the IPreC study setting [99, 101], and whether any biphasic dose-response between ischemic severity and protein expression exists related to neuronal survival or death has not been quantitatively examined in detail, nor shown in a hormetic model for IPreC study.

Why integrate the concept of hormesis into research on IPreC and IPostC?

As discussed, we cautiously accept hormesis as a general concept covering all patterns of IPreC and IPostC in stroke until thorough study and deep examination confirm that hormesis is compatible with IPreC and IPostC in stroke. Since the concepts of IPreC and IPostC have been extended to include a broad range of insults [22, 102, 103], we do not exclude the notion that some forms of preconditioning and postconditioning may fit perfectly into the hormetic context. Despite concerns, our enthusiasm for the concept of hormesis in stroke research remains undimmed [22]. We strongly encourage more well-designed studies to determine definitively if IPreC and IPostC are forms of hormesis. In addition, since hormesis is manifested by thousands of proven agents and chemicals [69-71, 74, 77], this may facilitate discovery of alternative tools for inducing preconditioning and postconditioning against stroke. We believe these results should be considered when candidates are chosen for stroke treatment. Hormetic data banks may provide invaluable clues for finding neuroprotectants in pre- and post-conditioning. Furthermore, even if the concepts of hormesis and IPreC and IPostC cannot be fully integrated, the quantitative features of hormetic models provide important methods to re-examine critical parameters of IPreC and IPostC. It is our hope that this review article will encourage the field to employ these features to inform decisions for clinical trials of IPreC and IPostC in stroke treatment.

As we discussed in a recent review article [22], whether or not IPostC or IPreC can be successfully translated to the clinic depends on several factors that must be assessed before a decision is made to commence IPreC or IPostC clinical trials. First, the scientific evidence for the protective effects of IPreC and IPostC in experimental stroke models must be presented, including optimal IPreC and IPostC paradigms, stroke models reflective of clinical stroke, and underlying mechanisms for these protective effects. Second, concerns of patients and medical doctors about the safety and efficacy of IPreC and IPostC must be addressed, including the risk of additional injury when one or more minor strokes are induced before or after a major stroke. Persistent fears exist regarding

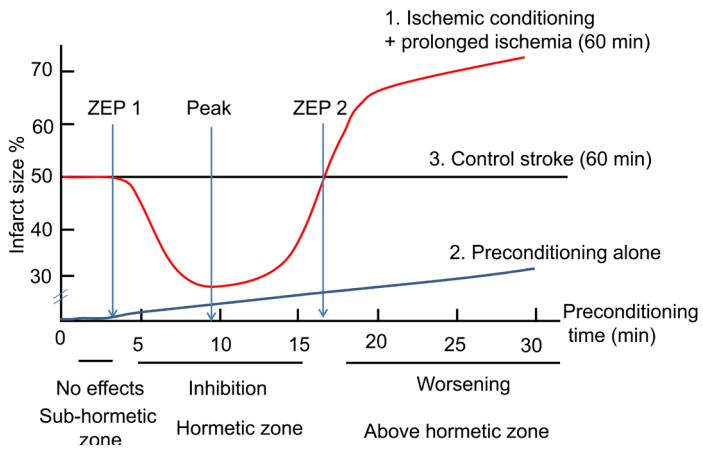

Figure 3. A typical hormetic pattern of the protective effects of IPreC against a test stroke. When a control ischemia is moderate, e.g., 60 min of MCA occlusion, IPreC may generate a typical "U" shaped-hormetic response. 1. Representative line of infarct sizes when IPreC is followed with a test ischemia. 2. IPreC alone shows gradually worsening effects on infarct sizes. 3. The line represents an infarct size when control ischemia alone is induced. ZEP, (zero equivalent point). In the sub-hormetic zone (below ZEP1), preconditioning has no protective effects. In the hormetic zone, preconditioning generate protection, and it is assumed a strongest protection can be measured. In the above hormetic zone (after ZEP2), preconditioning may worsen infarction. Control ischemia time (60 min) and preconditioning times for hormetic zones are arbitrary, the time points are given as references, which are not based on any experimental results.

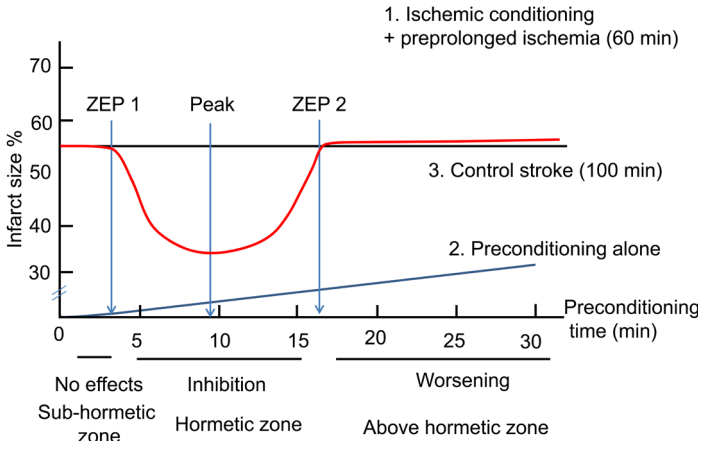

Figure 4. An alternative hormetic pattern of the protective effects of IPreC against a control stroke. When ischemia is greater in severity than the ischemia proposed in Figure 3, e.g., 100 min of MCA occlusion, IPreC may generate an atypical "U" shaped-hormetic response, in which a hormetic zone can still be identified, but an unfavorable, prolonged preconditioning may not be able to worsen infarction.

acceptance of IPreC and IPostC in the clinical setting. Below, we detail our strategies to evaluate both the safety zone and the effective zone of IPreC and IPostC by using the quantitative index of hormetic models.

Using hormetic strategies to identify the safe, effective and detrimental zones for IPreC and IPostC against stroke

Although IPreC or IPostC alone shows a threshold feature as a function of infarction or neuronal death, the combination of IPreC or IPostC plus a test ischemia may generate a typical hormetic pattern when measuring the protective effects. We predict this is dependent on ischemic conditioning periods or patterns, conditioning onset time, stroke models and stroke severity. Since we have discussed a hormetic strategy to study postconditioning in a recent review article [22], this review focuses on preconditioning.

In a typical hormetic pattern with representative J or U shaped profiles (**Figure 3**), three critical points are expected to be identified. Zero equivalent point ZEP1, peak point, and ZEP 2. In the case of IPreC, if IPreC is induced by a single period of brief ischemia, ZEP1 represents a point below which no protective effects are observed, and above which protective effects are detected. ZEP2 represents a point below which protective effects are observed and worsening effects are expected. A point between ZEP1 and ZEP2 is expected to be where the strongest protection occurs. After ZEP2, a combination of IPreC with a test ischemia (control ischemia) will produce a larger infarction and gradually reach a maximal infarction, thus reaching a plateau for the curve. For this situation, the test ischemia must be in a moderate range where brief preconditioning can reduce infarction, and prolonged preconditioning can worsen infarction, as we assume that when ischemia reaches a certain degree of severity, no amount of preconditioning can generate protective or detrimental effects on the final infarct sizes.

A second hormesis-like pattern of the protective effect of IPreC is that IPreC can generate

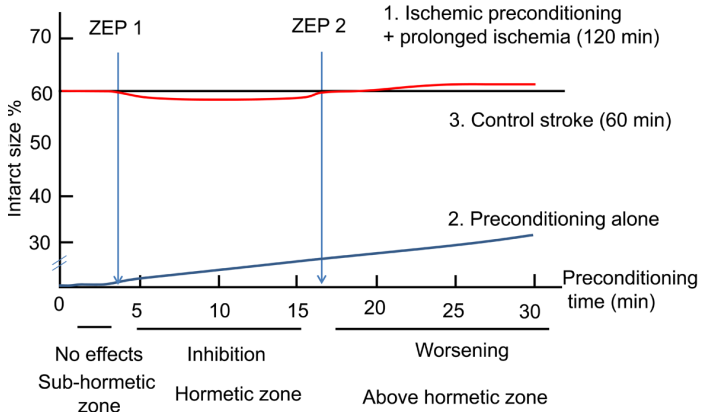

Figure 5. A third pattern of the effects of IPreC against a control stroke. When a test ischemia is too severe, e.g., 120 min or longer of MCA occlusion, IPreC may generate minimal protective or detrimental effects.

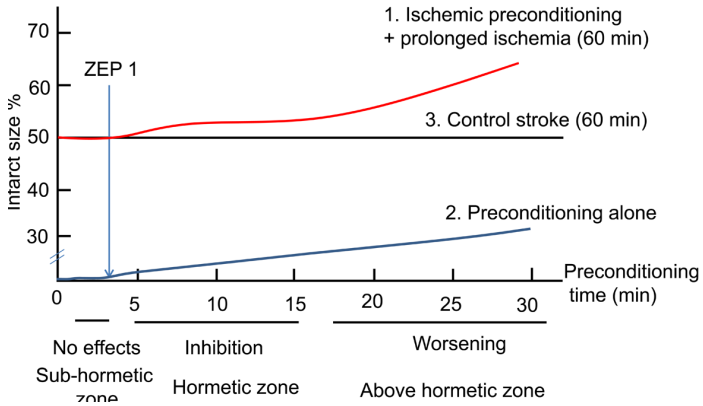

Figure 6. A fourth pattern of the effects of IPreC against a test stroke. When ischemia is moderate, e.g., 60 min or longer of MCA occlusion, IPreC may worsen ischemic outcomes performed within an unfavorable therapeutic time window.

protection, but after ZEP2, prolonged-IPreC does not generate protection or detrimental effects (**Figure 4**). This situation may happen when the control ischemia is moderate enough to allow preconditioning to generate protection, but severe enough to prevent a prolonged-preconditioning from further worsening infarction.

A third possibility is that IPreC generates neither protection nor aggravation of the subsequent ischemia (**Figure 5**). This may happen if the test ischemia is too severe for IPreC to alter the pathological outcomes. It is also possible that the ischemic brain does not respond to an IPreC when performed in an inappropriate time window.

Yet a fourth pattern may also exist (**Figure 6**), in which IPreC generates additional worsening effects to the subsequent prolonged-ischemia, thus larger infarct sizes may be detected. This may happen when using a moderate ischemia and conducting preconditioning in an unfavorable time window.

In the case of IPostC, the above 4 patterns of the final pathological outcomes may also exist. A major difference is that IPostC is performed after reperfusion, and is usually induced by a series of brief occlusions of the cerebral blood vessels. The number of occlusions, the period of occlusion, the onset time of IPostC, the severity of ischemia and the stroke models will influence which pattern occurs.

Summary and conclusion

Whether IPreC and IPostC in stroke belong in the hormetic context or not based on the quantitative index of hormetic models has not been examined in detail. According to current available evidence, IPreC alone without a subsequent severe ischemia will generate a threshold, but not a hormetic dose-dependent response, when infarct sizes are used as the endpoint measurement. Nevertheless, the dose-response relationship in an IPreC setting may fit hormetic models at molecular and cellular levels. Furthermore, IPostC may not match the classic hormetic model if the major function of IPostC is to interrupt reperfusion and attenuate oxidative stress, rather than as an additional stimulus to the ischemic brain. However, some patterns of preconditioning and postconditioning, which are induced by other neruoprotective agents, rather than ischemia, may perfectly match the criteria required for hormesis. These preconditioning and postconditioning patterns have not been carefully studied using a hormetic quantitative index. Regardless of whether there are conflicts or not between IPreC or IPostC and hormesis, the protective effects of IPreC and IPostC against brain injury in certain stroke models may show an ideal pattern of curve, identical to a hormetic model. We strongly advocate using the quantitative features of hormetic models to evaluate safe and effective

zones for the clinical translation of both IPreC and IPostC.

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