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ADDENDUM

An unexplored brain-gut microbiota axis in stroke

Shu Wen Wen and Connie H. Y. Wong

Centre for Inflammatory Diseases, Department of Medicine, School of Clinical Sciences, Monash University, Clayton, Victoria, Australia

ABSTRACT

Microbiota research, in particular that of the gut, has recently gained much attention in medical research owing to technological advances in metagenomics and metabolomics. Despite this, much of the research direction has focused on long-term or chronic effects of microbiota manipulation on health and disease. In this addendum, we reflect on our recent publication that reported findings addressing a rather unconventional hypothesis. Bacterial pneumonia is highly prevalent and is one of the leading contributors to stroke morbidity and mortality worldwide. However, microbiological cultures of samples taken from stroke patient with a suspected case of pneumonia often return with a negative result. Therefore, we proposed that post-stroke infection may be due to the presence of anaerobic bacteria, possibly those originated from the host gut microbiota. Supporting this, we showed that stroke promotes intestinal barrier breakdown and robust microbiota changes, and the subsequent translocation of selective bacterial strain from the host gut microbiota to peripheral tissues (i.e. lung) induces post-stroke infections. Our findings were further supported by various elegant studies published in the past 12 months. Here, we discuss and provide an overview of our key findings, supporting studies, and the implications for future advances in stroke research.

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Stroke promotes gut dysfunction and alters the gut microbiome

According to the recent Global Burden of Disease (GBD) study, stroke continues to be a significant health concern with an impact predicted to increase due to an aging population.[1] After ischemic and hemorrhagic stroke, up to 50% of patients experience gastrointestinal complications, including dysphagia, gastrointestinal hemorrhaging, constipation and bowel incontinence.[2-4] The underlying mechanisms may be attributed to the highly innervated nature of the intestinal tract by both extrinsic and intrinsic nerve fibers. The resulting gastrointestinal complications after stroke are associated with poor patient outcomes, including delayed patient recovery times, increased mortality rates and deteriorating neurologic function.[2,5-9] Despite this, a less well understood secondary effect of stroke is gut microbiota dysbiosis. Impaired intestinal microbiota balance is known to contribute to neuro-behavioral problems,[10-14] inflammatory disease states,[15-19] and more recently in pre-clinical settings, shown to worsen stroke outcomes.[20,21]

Stroke and transient ischemic attack patients often display significant changes in microbial diversity and bacterial counts in fecal samples independent of certain co-morbidities (age, hypertension and type 2 diabetes).[22,23] However, clinical studies remain limited when trying to delineate the underlying mechanisms for microbiota changes in these patients. The main challenge is the heterogeneous nature of patient clinical pathology, diets and lifestyle, all of which have major influences on the gut microbiome composition. From experimental studies, however, several potential mechanisms for microbiota imbalance after stroke has been proposed, including the suppression of systemic immunity,[24-28] the release of pro-inflammatory mediators from brain infarct lesions,[29,30] activation of the sympathetic nervous system (SNS),[26,31-33] stress induction,[34] and impaired intestinal motility.[20] It is likely a multitude of causative factors are simultaneously at play.

CONTACT Connie H. Y. Wong, PhD. connie.wong@monash.edu Centre for Inflammatory Diseases, Department of Medicine, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, VIC 3168 Australia.

Our recent experimental study adds substantially to this body of evidence.[33] In this study, we used a well-accepted and clinically relevant mouse model of cerebral ischemia-reperfusion injury: the intraluminal middle cerebral artery occlusion model (MCAO). This model involves the transient occlusion of blood supply to the middle cerebral artery in the brain using a monofilament with defined diameter. To understand the effect of stroke on the host microbiota, we first examined bacterial biomass and diversity in specific intestinal compartments after transient MCAO induction. We noted that bacterial biomass was considerable reduced at 24 hours after stroke onset in the ileum and colon, as well as significant alterations in mucosal microbiota composition at all taxonomic levels. This indicated selective depletion of specific bacterial strains, and/or possible translocation of bacteria away from the gut. Indeed, culturable bacteria was detected in the lung, broncho-alveolar lavage fluid (BALF), liver and spleen of stroke-induced mice compared with sham-operated cohorts, strongly suggesting systemic dissemination of gut bacteria. Sham-operated mice are essential in all MCAO models as it allows us to delineate if observed effects are directly due to stroke induction or simply because of surgical stress.

To assess if microbiota imbalance was associated with changes in intestinal function post-stroke, we examined gut permeability by quantifying serum levels of orally gavaged FITC-labeled dextran.[33] Vascular and epithelial permeability in the gut was significantly increased as early as 3 hours after stroke induction. Changes to gut permeability was further confirmed by the reduced distribution of ZO-1 tight junction complexes in the ileum, which are crucial in the maintenance of epithelial and endothelial barriers of the gut. It is unclear from our study whether other tight and adheren junctions known to regulate bacterial dissemination are altered after stroke, including occluding, cingulin, VE-cadherin and β-catenin.[35] Additionally, elucidating the effect of stroke on pericytes and fibroblasts (cells which are also associated with maintenance of gut vascular barriers) would be important to gain further insights into the overall post-stroke gut microenvironment.

Furthermore, our study revealed gut dysbiosis and intestinal dysfunction was associated with imbalanced sympathetic nerve signaling within the submucosal plexus of the gut following stroke.[33] Quantitative analysis of neuronal densities in the submucosal plexus of the ileum showed significant loss of cholinergic ChAT+ cells, and an overall imbalance between adrenergic and cholinergic signaling. Our findings are supported by a recent study that showed enteric neuronal loss after stroke in a galectin-3 and TLR4 mediated manner.[31] The administration of pharmacological β-adrenergic receptor inhibitors (propranolol or metoprolol) was able to restore stroke-induced gut permeability levels comparable to sham-operated mice, as well as reduce detectable bacteria in the lung, BALF, liver and spleen.[33] Consistent with our findings, a recent study demonstrated noradrenaline release after stroke altered the composition of the host microbiota, mucoprotein and goblet cell numbers in the cecal.[32] Besides effects in the gut, we have previously shown that noradrenergic innervation suppresses the ability of invariant NKT cells in the liver to effectively respond to bacterial infection after experimental ischemic stroke.[28] Taken together, these findings demonstrate the importance of β-adrenergic signaling in dictating stroke outcome, and present a potential therapeutic avenue to reduce stroke-associated complications in patients.

Other studies using a similar experimental stroke model confirm our findings. Singh et al (2016) showed stroke induces substantial changes to intestinal microbial composition, with an overall reduction in species diversity.[20] Specifically, alternations were observed within the highly abundant phyla: Firmicutes, Bacteroidetes and Actinobacteria.[20] This effect was associated with intestinal barrier dysfunction and reduced intestinal motility. Surgical manipulation of the ileus to mimic gastrointestinal paralysis patterns of stroke animals recapitulated similar changes in gut microbiome diversity.[20] Of note, the degree of intestinal dysfunction correlates positively with stroke severity,[20,36] indicating that the degree of brain infarct and neurologic impairment is key in determining systemic effects. Similarly, experimental traumatic brain injury in rats is associated with severe mucosal atrophy and disruption of gut epithelial cell tight junctions by 3 hours after injury, which can persist for 7 d.[37] Despite a consensus between multiple studies that gut dysfunction is essential to microbiota changes following stroke (or brain injury), much requires clarification. For example, what is the exact sequence of events mediated by this brain-gut-microbiome axis? Does microbiome dysbiosis precede changes in gut permeability, or does it occur simultaneously? Why are certain bacterial populations more prone to change after stroke, and are enteropathogenic strains more likely to persist and translocate to cause infection?

Gut dysbiosis after stroke regulates immunological changes

The brain-gut communication is bidirectional. Disruption of microbial-host symbiosis is linked to neurologic disorders, obesity, metabolic disorders and diabetes.[38-40] For example, a recent study elegantly demonstrated that the gut microbiota is essential for the development Parkinson disease in transgenic animals overexpressing human protein α-synuclein (αSyn).[41] In the absence of gut microbiota (germ-free conditions or antibiotics administration), transgenic animals display reduced motor deficits, microglial activation and αSyn aggregation in the brain compared with control animals with a complex microbiota.[41] Furthermore, colonisation of αSyn transgenic mice with microbiota from patients with Parkinson disease exacerbated motor dysfunction compared with mice that received microbiota from healthy controls.[41] In terms of stroke, emerging evidence suggests that a general loss of gut microbial diversity has cerebral effects.[20,42]

When intestinal microbial diversity was reduced using broad-spectrum antibiotics before stroke, brain infarct size decreased by 60%.[42] Antibiotics expanded members of Proteobacteria, and reduced in Firmicutes and Bacteriodetes.[42] This was associated with increased neuroprotective regulatory T cells in the gut, and its subsequent IL-10-mediated suppression of pro-inflammatory IL-17-positive $\gamma\delta$ T cells.[42] Conversely, post-stroke mice were shown to have larger brain infarct lesions and reduced behavioral performance if they were recolonized with altered gut microbiota obtained from stroke donors.[20] Recipients of stroke-induced microbiota also exhibited increased pro-inflammatory cytokines (IFN-γ and IL-17) in the brain and Peyer's Patch.[20] Importantly, it was shown using *in vivo* cell-tracking studies that fluorescently labeled T cells and monocytes in the Peyer's Patch migrate from the intestine to the brain after stroke, where they may cause tissue injury.[20] These studies demonstrate the complexity of intestinal flora, and the biomodial effects bacterial populations distal from the brain have on cerebral inflammation.[20,42] While difficult, extensive studies are required to define what neuroprotective and harmful gut bacterial species are important in stroke, and how they may be manipulated and harness therapeutically to improve patient outcomes.

Post-stroke infection can originate from endogenous gut bacteria

Despite its known primary effects of brain injury, the major cause of death after stroke is attributed by secondary infections, including pneumonia and urinary tract infections.[43] In fact, post-stroke infections account for up to 30% of mortality in patients.[44] Randomized clinical trials evaluating preventive antibiotics in patients with acute stroke showed it was not associated with reduced mortality.[45,46] Additionally, several large clinical studies have been unable to show a link between administration of antibiotics and reducing post-stroke infection.[45,47] This suggests a clear need for alternative treatment approaches and importantly, a better understanding of the underlying mechanisms of post-stroke infections. Current known risk factors for post-stroke infection include dysphasia, immobility, bulbar palsy and subsequent development of aspiration. Although these factors may play a role, our recent work demonstrated that a major source of lung infection post-stroke originates from gut commensal bacteria translocating systemically following the breakdown of gut barriers after stroke onset.[33]

Our recent findings showed that the majority (> 70%) of bacteria detected in stroke patients who developed infections were common commensal bacteria that normally reside in the intestinal tracts (*Enterococcus spp., Escherichia coli* and *Morganella morganii*).[33] Culturable bacterial colonies were absent from the blood of healthy control patients. To confirm our suspicion that bacteria originates endogenously from within the host gut, we turned to the MCAO mice model. Post-stroke infection was shown to be specific to mice raised in specific-pathogen-free (SPF), and not those in germ-free (GF) facilities after MCAO surgery. Interestingly, this effect was consistent despite similar brain infarct size between SPF and GF mice after stroke. Unlike SPF mice, those in GF conditions lack a normal gut microbiota, hence allowing us to directly pinpoint if bacterial infection after stroke is likely to originate from exogenous or endogenous sources. To further examine if post-stroke infection arise due to the translocation of host gut microbiota, streptomycin-resistant *E. Coli* was inoculated into SPF mice via oral gavage. *E. Coli* was strategically inoculated 3 hours after stroke induction, a time point which coincidences with increased gut permeability. We found only post-stroke mice displayed positive cultures of streptomycin-resistant *E. Coli* in the lung,

blood and liver compared with surgical sham controls, clearly indicative of gut bacteria dissemination systemically. Two major pathways of gastrointestinal permeability may be responsible for bacterial translocation: transcellular through the epithelial cells and/or paracellular past the tight junctions.[48] While we have evidence of bacterial translocation via paracellular means post-stroke (ZO-1 tight junction breakdown),[33] no studies to date has explored the movement of bacterial or bacterial components via transcellular pathways in a stroke setting. The transcellular release of bacterial components through the epithelial barrier could prove important in programming the underlying immune response imitated in the lamina propria after stroke.

To characterize the complete microbial community after stroke and expand beyond culture-based analysis, we utilised high-throughput 16S rRNA gene amplicon sequencing to analyze the lung microbiome from sham-operated and post-stroke SPF mice.[33] We showed that there were no differences in α diversity metrics between the lungs of sham-operated and post-stroke mice, but there were significant shifts in the abundance of existing microbiota at most taxonomic levels. Using bioinformatic algorithms (SourceTracker), we predicted the most likely origin of microbial communities present in the lung from post-stroke mice are the small intestine and liver. Future studies using super-resolution live-cell imaging technology would be useful in providing real-time tracking of bacterial movement away from the gut, and specificity when seeding in other tissues.[49] The ability to visualize and study the host-pathogen interaction at the mucosal surface will also provide insights into the underlying mechanisms behind the selectivity of bacterial translocation.

Concluding remarks

While considerable advances has been made in the last few years, it is clear from the recent publications summarized that much remains unanswered concerning the role of the gut microbiome in stroke. In particular, what is the key driver of bacterial gut translocation, is the dissemination strain specific or dependent on overall gut diversity, and once translocated, is bacterial colonization organ specific? Answers to these key questions may enable targeting of critical pathways to inhibit systemic bacterial dissemination and outgrowth in secondary organs. However, a major obstacle for the clinical translation of microbiota research is the large variation between the gut microbiome of patients (due to lifestyle, diet and co-morbidities), which cannot be easily replicated using mouse models. Detailed profiling and characterization of the microbiome using high throughput sequencing, metabolomics and computational methodologies will likely be important to assist in developing personalized treatment approaches. Nevertheless, microbiome-based interventions hold great potential for improving stroke outcomes.

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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