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RESEARCH ARTICLE
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EVIDENCE OF MENTHOL-MEDIATED AMMONIUM TRANSPORT VIA TRANSIENT RECEPTOR POTENTIAL (TRPV3 AND TRPM6) CHANNELS ACROSS ISOLATED RUMEN AND OMASUM EPITHELIA OF BUFFALO (*BUBALUS BUBALIS*)

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ABSTRACT
Fermentative protein degradation to ammonia and excretion of nitrogenous compounds from ruminants is a global environmental concern. Exploration of ammonia uptake mechanisms in the fore-stomach of ruminants is one of the approaches to tackle this issue. In cattle, channel-mediated ammonium uptake in the rumen is well established and occurs via transient receptor potential (TRP) channels with a sensitivity to essential oils. However, the expression, sensitivity, and role of these channels in the fore-stomach of buffaloes (*Bubalus bubalis*) remains to be ascertained. This study investigated the functional expression of TRP channels in indigenous buffalo's rumen and omasal epithelia and their involvement in menthol-mediated ammonia transport. Viable segments of rumen and omasum epithelia were collected immediately from slaughtered adult indigenous buffalo (N=8) for molecular expression of TRP channels. The isolated epithelia were mounted on Ussing chambers with mucosal buffer solutions containing NH_4Cl (0mM, 10mM, and 40mM and osmolarity 300, 350, or 400 mosmol·l^{-1}) with or without menthol. Results showed that TRPV3 and TRPM6 were expressed in both rumen and omasum epithelia; however, the expression was significantly lower in omasum. In vitro experiments with isolated ruminal and omasal epithelia exposed to varied mucosal ammonium buffer revealed a menthol-sensitive short-circuit current (rumen > omasum) and acidification of serosal buffer. An increase in mucosal osmotic pressure increased transepithelial tissue conductance, which was not influenced by mucosal menthol. This study suggests that TRP channels are expressed in the rumen and omasum epithelia of buffalo and are involved in menthol-sensitive ammonium transport. Further research on these channels could help mitigate ammonia release from livestock.
Keywords: Ussing chamber, ammonia, essential oils, short circuit current, TRP channels, ruminants

Introduction
Ruminants are unique in that they harbor various microbes in their fore-stomachs that can convert non-protein nitrogen, such as urea, for the de-novo synthesis of protein. In Pakistan, 42 million

buffaloes provide a primary source of milk and meat for human consumption. Offering urea-treated feed straw to ruminants in the subcontinent is typical for enhanced productivity [1]. However, ~72% of the nitrogen fed to ruminants is wasted in their excreta, predominantly as ammonia, which is linearly and positively correlated with nitrogen intake [2]. Escalated ammonia emission from livestock is a global concern as it degrades agricultural land and jeopardizes human food security [3].

It is now well established that at physiological ruminal pH (6.0 – 7.2), NH_3 predominantly occurs in its protonated form, i.e., NH_4^+. From recent studies on Holstein-Friesian cattle [4] and Sahiwal-mix cattle [5], it is evident that the apical uptake of ammonium occurs via the transient receptor potential family (TRP) while its basolateral route is via a non-selective cation channel. Although known for their sense of olfaction and temperature, TRP channels are expressed throughout the gut, and their role is still investigated. In the gut, various stimuli result in Ca_2^+ influx in the sensory neurons, initiating a signaling cascade for the perception of local response through the enteric nervous system [6]. Interestingly, TRP channels, known to enhance intestinal motility and digestion (TRPA1), are also expressed on intestinal epithelial cells as non-selective cation channels for the uptake of Ca_2^+ (TRPV5 and TRPV6) and Mg_2^+ (TRPM6) [7].

Essential oils (EO) are considered beneficial in ruminants [8, 9] as antimicrobials due to their capability to interfere with the deamination of amino acids in vitro rumen samples as well as their ability to modulate rumen fermentation [10]. Menthol is also known to invoke TRP channels in the rumen of cattle [4]. However, the literature regarding the presence of TRP channels in the forestomach of buffalos and their sensitivity to EO is scarce. This study investigates the novel expression of TRP channels in the rumen and omasum of Indigenous buffalo bulls and its possible involvement in menthol-sensitive ammonium transport.

Materials and Methods

Animal rearing and slaughtering

In this study, isolated ruminal and omasal epithelia were obtained from young indigenous buffalo bulls (N=8, Nili-Ravi breed) reared at a commercial fattening farm, Big Feed (Pvt.) Ltd. Lahore Pakistan. All the animals were raised under standard feed-lot fattening strategies and uniform pre-slaughter management practices. At 24 months, the animals (280 ± 15 kg) were transported to the University of Veterinary and Animal Sciences, Lahore, Pakistan, and slaughtered under veterinary supervision. The Animal Care and Use Committee UVAS-Lahore approved all the procedures followed in this study.

Isolation, preparation, and transport of tissue

Briefly, a ~200 cm^2 piece of ventral rumen and a few laminae of omasum were cleaned with a pre-warmed and gassed (95% O_2 and 5% CO_2) transport buffer solution that consisted of (mM): 115 NaCl, 25 $NaHCO_3$, 0.40 NaH_2PO_4, 2.40 Na_2HPO_4, 5 KCl, 5 Glucose, 1.20 $CaCl_2$, and 1.20 $MgCl_2$ [11]. The epithelium was stripped from the muscle layer, transported to the laboratory, and mounted on the Ussing chambers for in vitro investigations [4, 5]. For molecular biology, the stripped epithelia were immediately collected in RNA later® (Ambion, Austin, TX, USA) and stored at -70°C for later use.

mRNA expression of TRP channels

The integrity of isolated RNA was determined through denaturing agarose gel electrophoresis. Only those tissues that contained integrated RNA, having a denser 28S band compared to the 18S band, were further processed for cDNA synthesis and PCR. GAPDH was selected as the reference gene, while TRPA1, TRPV1, TRPV3, TRPM6, and TRPM7 were the target genes (Table 1).

Table 1: Primers used for expression analysis of TRP channels.

GENE	PRIMERS		Product Size(Base pairs)	Melting temperature ^{o}C
$TRPA_1$	**Fwd**	Gatgatgtgaatgcctca	157	58.6
	Rev	Ccacctggttttttcctgc		63.7
$TRPV_3$	**Fwd**	Gtgcagatgctgatggagaa	256	63.5
	Rev	Tgatctcacggctgagaatg		64.3
$TRPM_6$	**Fwd**	Acattggtctcctgcttc	141	56.9
	Rev	Acttttccacacactgttcttc		57.4
$TRPM_7$	**Fwd**	Tcaacaggcaggaccttatg	149	56.7
	Rev	Caagagtccaagatggtg		55.5
$TRPV_1$	**Fwd**	Cacgtacatcctcctgctca	225	61.5
	Rev	Gtgttccaggtggtccagtt		63.3
GAPDH	**Fwd**	Gacaagatggtgaaggtcg	102	62.0
	Rev	Cattgatggcgacgatgt		62.1

The primers reported for expression analysis of the target genes in cattle were used in the current study [4]. An in-silico comparative homology analysis of these primers was performed against the predicted target gene sequences in buffalo (Bubalus bubalis) to verify their usefulness (XM_006047233.4, XM_006079709.3, XM_044939956.2, XM_006077783.4, XM_006079707.3, XM_006065800.4). For expression analysis of the target genes, mRNA extraction from 50 mg each of rumen and omasum tissue samples was performed using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to synthesize complementary DNA. The reverse primer of each of the selected primer pairs was used for reverse transcription. Before cDNA synthesis, the total RNA concentrations extracted were determined using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific). For approximate quantitative gene expression analysis, the end-point PCR amplicons amount was compared to the concentration of nearby DNA bands in the standard marker (100bp Opti-DNA Marker, cat # G016) (Figure 1B). A three-step protocol was used for PCR amplification for the Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific). Each 20 µl PCR reaction contained 2 µl of cDNA (50 ηg), 10 µl of 10× buffer containing 200 µM of each dNTP and 1.5 mM of MgCl2, 1 µl of 10 ρmoles of each of forward and reverse primer, 0.25 µl of Phire Hot Start II DNA polymerase and 5.75 µl of ddH2O. A 27-cycle three-step PCR protocol (30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C) was performed on SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific). Reaction products were analyzed through 1.5% agarose gel electrophoresis. Quantity (ηg/µl) of PCR amplicons was estimated against 100bp Opti-DNA Marker (ABM, Richmond, BC, Canada) by comparing their amount with that of nearby DNA band in the marker (Figure 1B), with the geneQuant software integrated into gelLite Gel Documentation System (Cleaver Scientific, Rugby, Warwickshire, UK).

Ussing Chamber Experiments

Freshly isolated ruminal and omasal epithelia were mounted on the Ussing Chamber with an exposed surface area of 3.14 cm^2. The experimental buffer (mucosal) contained (mM): 70 NaCl, 30 Na-gluconate, 5 glucose, 5 KCl, 0.4 NaH_2PO_4, 2.4 Na_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$ 40 NMDGCl (N-methyl-D glucamine chloride) while the pH was adjusted to 6.8. In the serosal buffer, NH_4Cl was replaced by 10 or 40 mM NMDGCl, and the pH was maintained at 7.4. Where mentioned, the mucosal buffer was replaced with 0, 10, or 40 mM NH_4Cl instead of NMDGCl. For experiments to observe changes in serosal pH (measured at the beginning and after 1 hr of the experiment), NaH_2PO_4 and

Na_2HPO_4 were replaced by 5 mM NaCl to weaken the buffering capacity, and the epithelial tissues were treated with either menthol (1 mM, dissolved in ethanol at a ratio of 1:1000 on each experimental day and kept on ice until added) or ethanol (1 mM) on the mucosal side. For experiments with the tonicity of the mucosal solution, the mucosal buffer was adjusted to 300, 350, and 400±2 mosmol·l^{-1} by mannitol, and the change in tissue conductance (Gt) was observed. The electrophysiological parameters were measured in short circuit mode by a computer-controlled voltage clamp device (Mussler, Aachen, Germany) where a cation transport from mucosal to serosal side produced a positive current, Isc.

Statistical Analysis

The data were subjected to the Schapiro-–Wilk test to ascertain normality and presented as mean ± SE. One-way ANOVA or Independent T Test, as appropriate, were employed using Sigmaplot (Systat Software Inc., UK, Version 11.0). Tukey's post hoc test was carried out for pairwise comparison between the treatment groups, and $p < 0.05$ was considered significant. Where applicable, 'N' refers to the number of animals, while 'n' refers to the number of epithelia.

Results

Expression of mRNA:

Both the ruminal and omasal epithelia expressed TRPM6 and TRPV3. Overall, the mRNA expression was markedly higher in the rumen compared to the omasum (Fig. 1), with significant differences in TRPM6 ($p < 0.001$) and TRPV3 ($p < 0.002$).

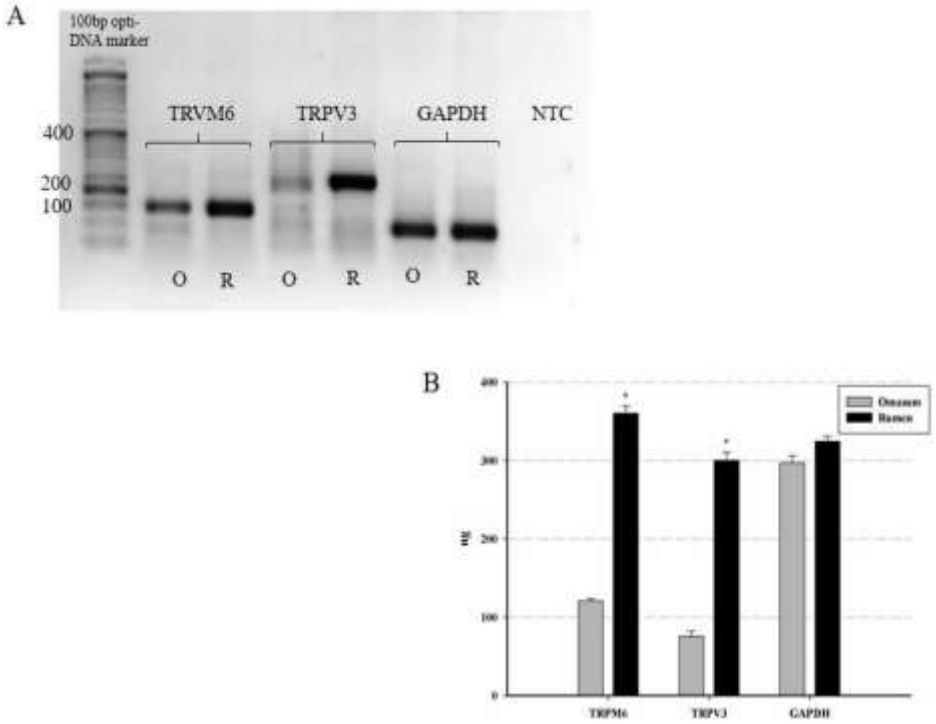

Figure 1: Expression of TRP in rumen and omasum epithelia of indigenous buffalo. 1A; Concentration of DNA bands in 100bp opti-DNA Marker in ng/5µl. 1B; Mean ± SE with significant difference* at $p < 0.05$ (N/n = 8/16)

The results of Ussing chamber experiments with NH_4Cl buffer showed that buffaloes' rumen and omasal epithelia exhibited a concentration-dependent, menthol-sensitive, ammonium-mediated modulation of short-circuit current (Isc). Here, we report for the first time that the menthol-sensitive electrophysiological response (Isc) was more significant in the rumen epithelium than in the omasum

epithelium (Figure 2A). The addition of ethanol (1 mM) to the mucosal side (10 mM NH_4Cl) did not elicit any response in the Isc (Figure 2B).

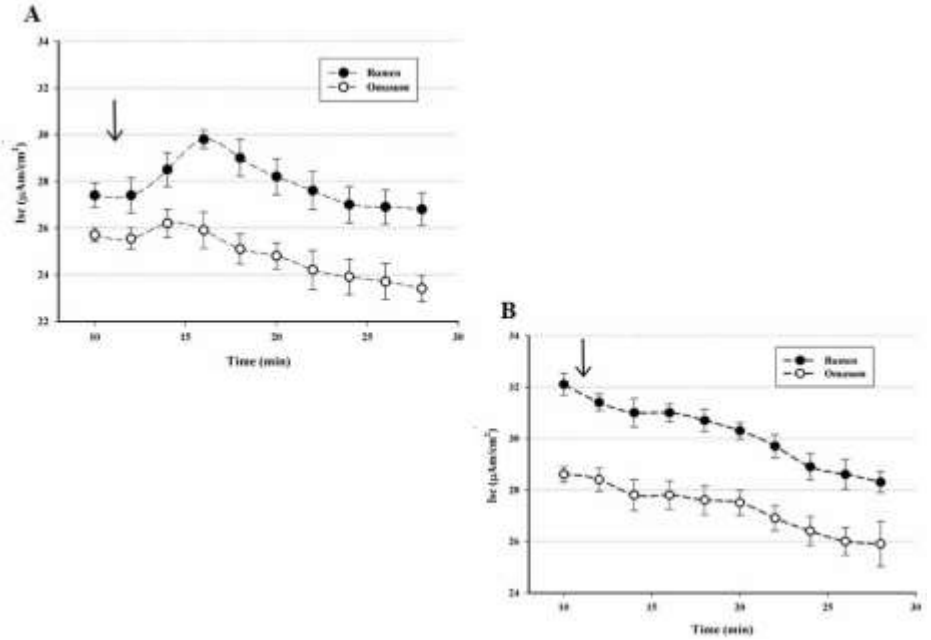

Figure 2: Real-time Isc recordings showing a response of mucosal addition (arrow) of menthol (A) or ethanol (B) in ruminal or omasal epithelia (N/n = 4/8)

To further ascertain the transport of ammonium from the mucosal to the serosal side, the serosal pH was monitored at the beginning and the end (1 hr) of incubation with either 0 mM, 10 mM, or 40 mM NH_4Cl buffer on the mucosal side with menthol (1 mM) added after 10-12 min of incubation. Figure 3 shows the acidification of serosal buffer from 7.4 to 7.1 (40 mM mucosal) in rumen epithelium ($p < 0.05$). The effect of ammonium transport on the serosal pH for omasum was non-significant for all three concentrations.

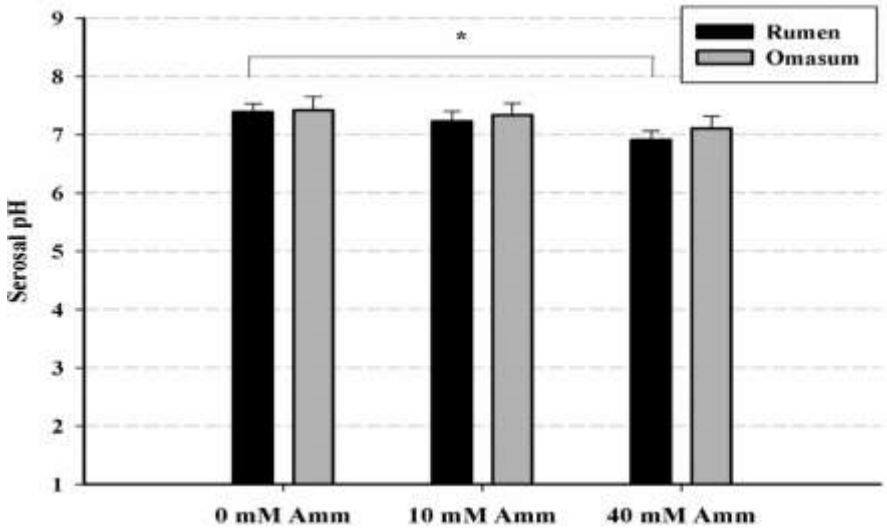

Figure 3: Effect of varying concentrations of NH_4Cl mucosal buffer pre-treated with menthol (1 mM, mucosal) on the serosal pH $_{(1hr)}$. (N/n = 8/14)

To assess the role of TRP channels in epithelial barrier functions, rumen, and omasum epithelia were exposed to mucosal buffers with various osmolarity. An increase in osmolarity increased the tissue conductance (Gt) linearly (400 > 350 > 300), and this change was depicted within 1-2 minutes, reaching stability within 20-30 minutes. This increase was more pronounced for the rumen compared

to the omasum epithelia. Adding menthol on the mucosal side did not affect Gt ($p > 0.05$) compared to the epithelia without menthol for all three osmolarity (Figure 4).

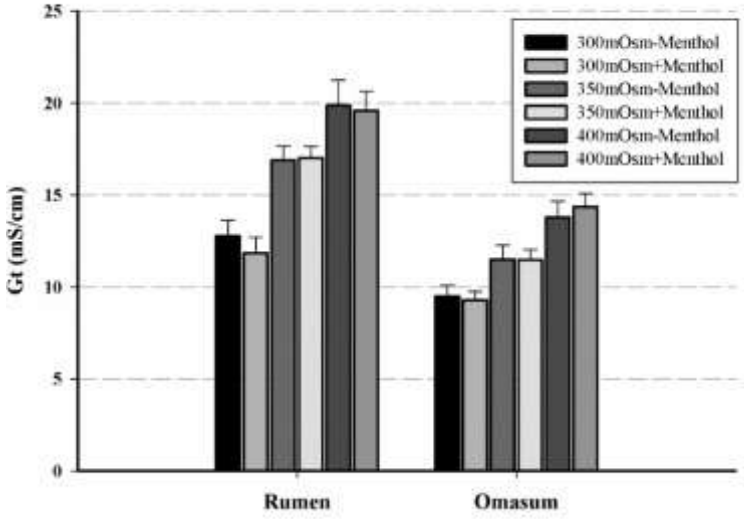

Figure 4: Effect of varying mucosal osmolarity with or without menthol (1mM, mucosal) on the tissue conductance in ruminal or omasal epithelia (N/n = 8/14)

Discussion

The current study aimed to determine the expression of TRP channels and their functional role in ammonia transport across isolated ruminal and omasal epithelia of indigenous buffalo. Viable segments of ruminal and omasal epithelia were subjected to various in vitro conditions to elicit the functional role of TRP channels in ammonium uptake. The presence of TRP channels in buffalo's rumen and omasal epithelia suggests their role in gut homeostasis, pH regulation, and cation transport, as reported previously [6]. Interestingly, the expression of TRP channels was significantly lower in the omasum compared to the rumen (Figure 1A), presumably because the rumen is primarily the leading site for electrolyte absorption [12], which is usually carrier-mediated [13-15]. The DNA bands for the remaining target genes were not detected, most probably due to a lack of expression of these genes in the studied tissues in Buffalo. These results contradict previous studies that reported their expression in cattle [16]. The lack of expression of these genes in buffalo cannot be attributed to the use of reported primers or optimization failure, as these issues were thoroughly addressed. All primer sequences were 100 % homologous to those of predicted gene sequences in buffalo except the forward primer sequence of each TRPA1 and TRPM6 gene. There were 1bp differences in these primer sequences between cattle and buffalo (TRPA1; Fwd cattle: gatgatgtgaatgcctca, TRPA1; Fwd buffalo: gatgatgtgaatgcctcg and TRPM6; Fwd cattle: acattggtctcctgcttc, TRPM6; Fwd buffalo: acattggtctcctacttc). The impact of single base pair difference in primers and template was nullified by analyzing the gene expression using multiple different PCR amplification temperature profiles and new primers without any mismatch. There was no difference in gene expression levels in any case. The amplification of the remaining TRP channels was not possible with the reported primers used in the current study. These primers have been designed to analyze TRP channels mRNA expression in Holstein-Friesian cattle. We decided to use the same primers for the buffalo genome due to the unavailability of the sequence data for the target genes in buffalo. The failure to specifically amplify the two TRP channel genes was probably due to the lack of homology at primer binding sites between cattle and buffalo.

The mucosal addition of menthol evoked a short circuit current that was more pronounced in the rumen than in the omasum. This suggests regional differences in TRP channel expression and their

involvement in ammonium transport in various gut segments, as reported by D. Manneck; H. S. Braun [17]. These findings also agreed with the previously reported studies [4, 5]. Furthermore, the response of Isc with menthol likely to be from the Cl^- can be ruled out since the apical transport of Cl^- is an electroneutral process that exchanges HCO_3^- and the resultant efflux of Cl^- from the basolateral side via an anion channel is less pronounced in rumen compared to colon [18]. Additionally, the serosal buffer's acidification in response to menthol's mucosal addition in the presence of NH_4Cl buffer illustrates the liberation of H^+ ions.

In ruminants, ruminal fluid varies in osmotic pressure depending on the diet and feeding practices. It becomes slightly hypertonic after feeding [19]. Increased osmolarity on mucosal buffers (350 and 400 mOsm) increased tissue conductance within 1-2 minutes. The increase in paracellular spaces due to the shrinkage of cells makes it easier for ions to flow between the cells. Similar trends were observed by U. Lodemann and H. Martens [11] for isolated ruminal epithelia of sheep in which the animals were offered hay with a supplemented diet, and the authors attributed the change in Gt to the paracellular pathway. In the present study, a non-significant change in the Gt upon the addition of menthol within the same tonicity validates the increment to be of paracellular origin. However, the involvement of ions in this increased tonicity-induced conductance was not the scope of this study.

Conclusion

This study showed that the rumen and omasum epithelia expressed TRPV3 and TRPM6 channels. The expression of these channels was linked to the physiological role of these tissues in the uptake of ammonia and perhaps other electrolytes that is more prevalent in the rumen than the omasum. In Ussing chamber experiments, acidification of the serosal buffer in the presence of menthol suggests the possibility of cation transport, primarily ammonium. Mucosal hyper-osmolarity increased transepithelial tissue conductance, which was not influenced by mucosal menthol. The findings of this research may open avenues to investigate further the role of TRP channels in mitigating ammonia release from livestock.

Author's Contributions

IR, MI, and MSY developed the original hypotheses and designed the experiments. SA conducted the research trial and collected the data for the experiment. IR, MSY, and MI performed the statistical analyses, interpreted the results, and wrote the initial draft of this manuscript. IR, MSY, and HR finalized the manuscript. All authors read and approved the manuscript.

Conflict of Interest Declaration

The authors declare that they have no conflict of interest.

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