在體內及體外探討海藻萃取物對於感染流感病毒的影響

盧文婷 (5136)

2020/09/30

大綱

摘要

流感病毒 (Influenza virus) 每年造成全球大流行，具有高發病率及死亡率。目前國內使用的抗流感病毒藥劑主要為克流感 (Tamiflu) 及瑞樂沙 (Relenza)，然而不良的副作用限制了這些藥物在治療流感中的用途，因此需要去開發新的抗流感藥物。故本篇報告的目的為探討海藻萃取物是否具有抗流感病毒的功效。籠目褐藻中的褐藻醣膠，能夠抑制神經胺酸酶 (Neuraminidase, NA) 的活性，乾擾表皮生長因子受體 (Epidermal growth factor receptor, EGFR) 途徑的活化，以限制 A 型流感病毒的釋放和抑制宿主細胞對 A 型流感病毒的內吞作用，並降低小鼠體內的病毒含量。螺旋藻萃取物為安全且耐受性良好，能透過降低病毒表面血球凝集素 (Hemagglutinin, HA) 的活性來抑制流感病毒，並且可以顯著抑制多種流感病毒的感染和複製，且能提升感染流感病毒小鼠的存活率。青海菜中的硫酸鼠李聚醣能抑制流感病毒感染的前期步驟，並增加抗體的產生，還能保護免疫正常和免疫功能低下的小鼠，免受 A 型流感病毒致命的攻擊。綜合上述的海藻萃取物，在體外能有效阻斷流感病毒感染，在體內能改善小鼠存活率並降低病毒量，具有發展成治療人類感染流感病毒療法的潛力。

一、 前言

流感病毒 (Influenza virus) 是全世界人類最重要的病毒性呼吸道感染源，可能導致呼吸道疾病和急性肺損傷的嚴重流行 (Cheng *et al*., 2012)。由於流感病毒基因的高突變率，有效的病毒傳播，耐藥性的迅速出現以及目前可用療法的有限效力，A 型流感病毒感染有可能導致更加危險的疾病 (Ding *et al*., 2014)。A 型流感病毒又可因表面的血球凝集素 (Hemagglutinin, HA) 以及神經胺酸酶 (Neuraminidase, NA) 的不同，而分成許多亞型，如 H1N1 和 H3N2。目前國內使用的抗流感病毒藥劑主要為克流感 (Tamiflu) 及瑞樂沙 (Relenza)。而克流感為口服式抑制劑，瑞樂莎為吸入式噴劑，皆為神經胺酸酶抑制劑。然而，不良的副作用，快速出現的抗病毒耐藥性，供應不足和價格高昂限制了這些藥物在預防和治療流感中的用途 (Yu *et al*., 2016)。因此科學家正積極尋找新的抗流感藥物。

海藻是生長在海洋中的藻類，依照內部所含的色素，可分為藍綠藻、綠藻、紅藻、褐藻 (Sureshbabu *et al*., 2015)。由於其生物多樣性，海藻是含有多種生物活性化合物的豐富天然資源，例如多酚，硫酸化多醣，萜烯，生物鹼，類胡蘿蔔素，固醇，蛋白質和抗氧化劑 (Hamed *et al*., 2015)。有許多研究指出，食用海藻能夠降低許多疾病的發生率，如糖尿病，肥胖，心血管疾病，乳癌，肺癌，人類免疫缺陷病毒等多種疾病 (Brown *et al*., 2014)。

因此本篇報告想要探討的主題為海藻萃取物是否具有抗流感病毒的功效。

二、 褐藻醣膠透過限制病毒神經氨酸酶和細胞 EGFR 途徑來抑制 A 型流感病毒的感染

籠目褐藻 (*Kjellmaniella crassifolia*) 為主要分佈於日本海域的大型褐藻，將其以熱水進行萃取，經過陰離子交換層析，分離出褐藻醣膠 (Fucoidan, KW)。利用核磁共振光譜法 (Nuclear Magnetic Resonance, NMR) 和電噴灑游離質譜法 (Electrospray Ionization Mass Spectroscopy, ESI-MS) 分析褐藻醣膠的結構，結果顯示褐藻醣膠的結構為多醣主鏈的 3 號碳上連接支鏈，支鏈上的 2, 4 號碳上接岩藻寡糖的葡萄糖醛甘露聚醣 (圖 1A) (Wang *et al*., 2017)。

- *In vitro*

細胞實驗的部分，以褐藻醣膠對犬腎細胞進行細胞毒性測試，再將犬腎細胞感染四種 A 型流感病毒，測試褐藻醣膠是否具有抗病毒的作用。將人類肺癌細胞感染 A 型

流感病毒後，觀察是否抑制了表皮生長因子受體 (Epidermal growth factor receptor, EGFR) 途徑。

在 62.5 至 1000 μg/ml 的濃度下，褐藻醣膠對細胞均無明顯的毒性 (圖 1B) (Wang *et al*., 2017)，且濃度越高抑制細胞病變的效果越好 (圖 1C) (Wang *et al*., 2017)。犬腎細胞和人類肺癌細胞在感染 A 型流感病毒後，使用褐藻醣膠治療能降低病毒表面蛋白 HA 的含量，表示褐藻醣膠對 A 型流感病毒的抑製作用並非細胞特異性，且能抑制 A 型流感病毒的複製 (圖 1D) (Wang *et al*., 2017)。以結晶紫染色計算病毒斑數量，結果顯示褐藻醣膠在 31.25 至 250 μg/ml 的濃度下減少了病毒斑數量，保護了犬腎細胞 (圖 1E) (Wang *et al*., 2017)。接著探討褐藻醣膠對各種品系 A 型流感的抑制作用，隨著濃度增加，HA 含量及病毒量均以劑量依賴性的方式降低，表示褐藻醣膠對於各種 A 型流感病毒和 HA 都有抑制效果 (圖 2) (Wang *et al*., 2017)。NA 為病毒表面的蛋白神經胺酸酶，會促進病毒的擴散，以褐藻醣膠治療能有效降低 NA 的活性，表明防止了病毒的擴散 (圖 3) (Wang *et al*., 2017)。

Eierhoff *et al*. (2010) 的研究指出活化 EGFR 路徑會增強細胞對 A 型流感病毒的吞噬作用。添加褐藻醣膠的組別，抑制了 EGFR 的活性還有內在化作用，且 EGFR 路徑的下游蛋白 PKCα、Akt、NF-κB 的表現量也下降，進而抑制 A 型流感病毒被宿主細胞吞噬 (圖 4) (Wang *et al*., 2017)。

- *In vivo*

使用雌性 ICR 小鼠進行兩種動物實驗，均分為以下 5 個組別：正常組，H1N1 組，H1N1 + 10 μg 褐藻醣膠組，H1N1 + 20 μg 褐藻醣膠組和 H1N1 + 20 mg/kg 克流感組。第一種為存活率實驗，每組 10 隻，在第 0 天用 1000 PFU 的病毒液攻毒小鼠，之後每天管餵一次樣品或藥物，持續 14 天，紀錄存活率至第 14 天。第二個實驗為由 H1N1 引起的急性肺損傷實驗，每組 4 隻，在第 0 天用 500 PFU 的病毒液攻毒小鼠，之後每天管餵一次樣品或藥物，持續 4 天，在第 4 天進行犧牲，採下肺臟和脾臟進行後續分析。

用褐藻醣膠治療 4 天後，與 H1N1 組相比，肺部病毒量顯著降低 (圖 5A) (Wang *et al*., 2017)。存活率實驗的部分，持續餵食褐藻醣膠 14 天，小鼠的存活率由 30% 提升至 80% (圖 5B) (Wang *et al*., 2017)。使用 HE 染色觀察肺部病理變化，H1N1 組的肺組織中有大量炎性細胞浸潤，以及管腔中存在大量漿細胞分泌物，使用褐藻醣膠治療後

減輕了肺部發炎的症狀 (圖 5C-G) (Wang *et al*., 2017)。最後測定了脾臟中抗病毒相關的細胞激素，結果顯示與 H1N1 組相比，用褐藻醣膠治療 4 天後，脾臟中 IFN-γ 和 IL-2 的產生顯著增加，這表明褐藻醣膠能夠過增強小鼠的免疫系統來抑制 A 型流感病毒複製(圖 5H & I) (Wang *et al*., 2017)。

綜合以上結果，籠目褐藻中的褐藻醣膠在體外能有效阻斷 A 型流感病毒感染，並且能夠抑制 NA 的活性以限制 A 型流感病毒的釋放，還能乾擾 EGFR，PKCα，NF-κB 和 Akt 的活化，抑制了宿主細胞在感染 A 型流感病毒時的內吞作用。在動物實驗中，以褐藻醣膠治療可以顯著提高小鼠存活率並降低病毒含量。

三、 耐受良好的螺旋藻萃取物抑制了流感病毒的複製並降低由病毒誘導的死亡率

螺旋藻 (*Arthrospira platensis*) 是一種自由漂浮的絲狀藍綠藻，含有 60-70% 的蛋白質含量和高濃度的酚酸，維生素 E，必需脂肪酸和維生素 B (Chen *et al*., 2016)。將其以冷水進行萃取，獲得螺旋藻萃取物，進行細胞及動物實驗，評估了螺旋藻萃取物的抗病毒特性。

- *In vitro*

使用犬腎細胞感染多種流感病毒後，探討螺旋藻萃取物是否具有抗流感病毒的作用。將豚鼠紅血球與病毒液混和，以血球凝集實驗來判斷，螺旋藻萃取物是否能抑制 HA 的活性。

分別以兩種 A 型流感 (H1N1 及 H3N2)、B 型流感、兩種對克流感具有抗藥性的 H1N1 以及 H7N9 感染犬腎細胞，經過螺旋藻萃取物治療後，均以劑量依賴性的方式抑制各種病毒株的病毒斑生成 (圖 6) (Chen *et al*., 2016)。在不同時間點加入螺旋藻萃取物，結果顯示在感染後的 3 小時以前加入萃取物，都能有效抑制病毒的複製，其中感染前一小時及感染後立即加入的組別，抑制了 90% 的病毒量 (圖 7) (Chen *et al*., 2016)。將螺旋藻萃取物與病毒液混和兩小時後，再加入細胞培養，顯著的抑制了病毒斑的生成 (圖 8A & B) (Chen *et al*., 2016)。而感染前一小時加入，感染後一小時移除樣品的組別，病毒量只有病毒控制組的 48%，表明螺旋藻萃取物可能直接影響流感病毒，而不是啟動宿主細胞的防禦機制來防止病毒感染 (圖 8C & D) (Chen *et al*., 2016)。接著選擇兩種 A 型流感 (H1N1 及 H3N2) 及 B 型流感與豚鼠紅血球混和，觀察血球凝集的情形，單獨病毒的組別，紅血球發生了凝集現象，表示病毒表面的 HA 沒有被抑制，而加入了

螺旋藻萃取物的組別，只要濃度大於 0.78 mg/ml 就能抑制 HA 的作用，表示能夠阻斷病毒複製的初期階段 (圖 8E) (Chen *et al*., 2016)。

- *In vivo*

使用 Sprague-Dawley (SD) 大鼠進行急毒性及亞急毒性試驗，評估螺旋藻萃取物的安全性。使用雌性 BALB/c 小鼠進行存活率實驗，分為以下 6 個組別：正常組，螺旋藻萃取物組，H1N1 組，H1N1 + 低、中、高 (10, 25, 50 mg/kg/day) 劑量的螺旋藻萃取物組，每組 5 隻，在第 0 天用 2×10^4 PFU 的病毒液攻毒小鼠，之後每天管餵兩次樣品，持續 4 天，觀察存活率至第 14 天。

已知螺旋藻萃取物在細胞實驗中具有抗流感活性，而在動物實驗的結果中，急毒性及亞急毒性試驗，無論是雄性或雌性大鼠的體重，所有劑量的螺旋藻萃取物組都跟控制組無顯著差異 (圖 9) (Chen *et al*., 2016)。將亞急毒性試驗的大鼠犧牲後，取血液進行分析，各項數據與正常的組別都沒有顯著差異，這說明即使在短時間內給予高劑量的螺旋藻萃取物也是安全且耐受性良好 (表 1) (Chen *et al*., 2016)。存活率實驗的部分，餵食 10、25、50 mg/kg 劑量螺旋藻萃取物的組別，小鼠的存活率由 0% 分別提升至 20%、40% 和 60%，代表螺旋藻萃取物提高了感染流感病毒的小鼠的存活率 (圖 10) (Chen *et al*., 2016)。

綜合以上結果，螺旋藻萃取物透過降低病毒表面 HA 的活性來抑制流感病毒，並且可以顯著抑制多種流感病毒的感染和複製。在動物實驗中，螺旋藻萃取物是安全且耐受性良好的，且能提升感染流感病毒小鼠的存活率。

四、 綠藻 *Monostroma nitidum* 的硫酸鼠李聚醣在正常和免疫力低下小鼠中的抗 A 型流感病毒活性

青海菜 (*Monostroma nitidum*) 是一種綠藻，生長在日本沿海的淺水區。將其以熱水進行萃取，經過陰離子交換層析，分離出硫酸鼠李聚醣 (Rhamnan sulfate, RS)，進行細胞及動物實驗，分析 RS 在體內及體外對感染 A 型流感病毒的影響。

- *In vitro*

使用犬腎細胞感染 A 型流感病毒後，探討 RS 是否具有抗病毒的作用。

於不同時間點加入 RS，在病毒感染期間加入 RS 的組別抑制效果最好，推測可能是抑制了病毒複製階段的前期，病毒吸附到宿主細胞或是宿主細胞啟動吞噬作用的階段 (圖 11) (Masahiro *et al*., 2020)。接著進行了病毒吸附分析及病毒入侵分析，當 RS 的劑

量越高抑制的效果越好，並且在同樣的時間裡，有加入 RS 的組別，病毒量比病毒組還要少，表示病毒的吸附及入侵，都有被 RS 抑制 (圖 12) (Masahiro *et al*., 2020)。

- *In vivo*

使用雌性 BALB/c 小鼠進行動物實驗，分為以下 6 個組別：H1N1 組，H1N1＋5 mg/day RS 組，H1N1 + 0.2 mg/day 克流感組，以及上述三組再加上 5-Fluorouracil (5-FU)。在第 0 天用 2×10^5 PFU 的病毒液攻毒小鼠，從感染 7 天開始每天管餵兩次樣品或藥物，持續 14 天，觀察存活率至第 14 天，分別在第 3、7、14 天進行犧牲，取其肺臟和血液進行後續分析。

免疫力正常的小鼠，在感染病毒後以克流感治療，體重完全沒有下降，而餵食 RS 的組別跟病毒組沒有明顯的差別 (圖 13A) (Masahiro *et al*., 2020)。5-FU 是一種抗癌藥物，會造成免疫力低下 (Kanoh *et al*., 2004)，誘導免疫力低下的小鼠，在感染病毒後以克流感治療，體重只有下降 5%，且在第 10 天之後就恢復，而餵食 RS 的組別，體重回復的現象比病毒組好一些 (圖 13B) (Masahiro *et al*., 2020)。在小鼠存活率的部分，只有免疫力低下的病毒組小鼠有兩隻死亡，其餘組別皆沒有，顯示餵食 RS 及克流感提升了小鼠的存活率 (表 2) (Masahiro *et al*., 2020)。犧牲小鼠後取肺沖提液進行分析，在病毒感染的 3 天後，無論有沒有誘導免疫低下，RS 組與病毒組的病毒量差異不大；而在感染 7 天後，免疫力正常的小鼠，只有感染病毒的組別檢測到病毒含量，餵食 RS 和克流感的組別，都檢測不到病毒含量，免疫低下的小鼠，餵食 RS 後病毒量顯著的下降，表示 RS 對免疫功能正常和免疫低下的小鼠均具有抑制 A 型流感病毒複製的作用 (圖 14) (Masahiro *et al*., 2020)。最後測試血清中的抗體含量，與病毒組相比，使用克流感治療會減少抗體的產生；而使用 RS 治療，能使血液中的抗體量上升，表示 RS 刺激了 A 型流感病毒抗體的產生 (圖 15) (Masahiro *et al*., 2020)。

綜合以上結果，硫酸鼠李聚醣能抑制流感病毒感染的前期步驟，並增加抗體的產生。還能保護免疫正常和免疫功能低下的小鼠，免受 A 型流感病毒致命的攻擊。

五、 結論

1. 海藻萃取物能抑制多種流感病毒的感染。
2. 海藻萃取物也能結合神經胺酸酶及血球凝集素去抑制 A 型流感病毒的複製。
3. 在感染流感病毒的小鼠中，給予海藻萃取物可改善存活率並降低病毒量。
4. 海藻萃取物具有發展成治療人類感染流感病毒療法的潛力。

參考文獻

Brown, E. M., Allsopp, P. J., Magee, P. J., Gill, C. I., Nitecki, S., Strain, C. R., McSorley, E. M. (2014). Seaweed and human health. *Nutrition Reviews*. 72(3), 205-216.

Chen, Y. H., Chang, G. K., Kuo, S. M., Huang, S. Y., Hu, I. C., Lo, Y. L., Shih, S. R. (2016). Well-tolerated Spirulina extract inhibits influenza virus replication and reduces virus-induced mortality. *Science Reports.* 6, 24253.

Cheng, V. V. C., To, K. K. W., Tse, H., Hung, I. F. N., Yuen, K. Y. (2012). Two years after pandemic influenza A/2009/H1N1: what have we learned. *Journal of Clinical Microbiology*. 25, 223-263.

Ding, Y., Dou, J., Teng, Z. J., Yu, J., Wang, T. T., Lu, N., Wang, H., Zhou, C. L. (2014). Antiviral activity of baicalin against influenza A (H1N1/H3N2) virus in cell culture and in mice and its inhibition of neuraminidase. *Archives of Virology*. 159, 3269-3278.

Eierhoff, T., Hrincius, E. R., Rescher, U., Ludwig, S., Ehrhardt, C. (2010). The epidermal growth factor receptor (EGFR) promotes uptake of influenza a viruses (IAV) into host cells. *PLOS Pathogens*. 6, e1001099.

Hamed, I., Özogul, F., Özogul, Y., Regenstein, J. M. (2015). Marine bioactive compounds and their health benefits: A Review. *Comprehensive Reviews in Food Science and Food Safety*. 14(4), 446-465.

Kanoh, K., Shimura, T., Suzuki, H., Nomoto, K., Asao, T., Kuwano, H. (2004). Antitumor effect of a splenic injection of 5-fluorouracil on metastatic liver cancer in mice. *Journal of Pharmacology and Experimental Therapeutics*. 308(1), 168-174.

Masahiro, T., Kyoko, H., Lee, J. B., Kaoru, N., Koichi, M., Toshimitsu, H., Toshio, K. (2020). Anti-influenza A virus activity of rhamnan sulfate from green algae *Monostroma nitidum* in mice with normal and compromised immunity. *Marine Drugs*. 18(5), 254-267.

Sureshbabu, N., & Manikannan, M. (2015). Emerging novel anti HIV biomolecules from marine algae: an overview. *Journal of Applied Pharmaceutical Science*. 5(9), 153-158.

Wang, W., Wu, J. D., Zhang, X. S., Hao, C., Zhao, X. L., Jiao, G. L., Shan, X. D., Tai, W. J., Yu, G. L. (2017). Inhibition of influenza A virus infection by fucoidan targeting viral neuraminidase and cellular EGFR pathway. *Science Reports.* 7, 40760.

Yu, J., Wang, D. H., Jin, J., Xu, J., Li, W. H., Wang, H., Dou, J. (2016). Antiviral activity of SA-2 against influenza A virus in *vitro/vivo* and its inhibition of RNA polymerase. *Antiviral Research*. 127, 68–78.

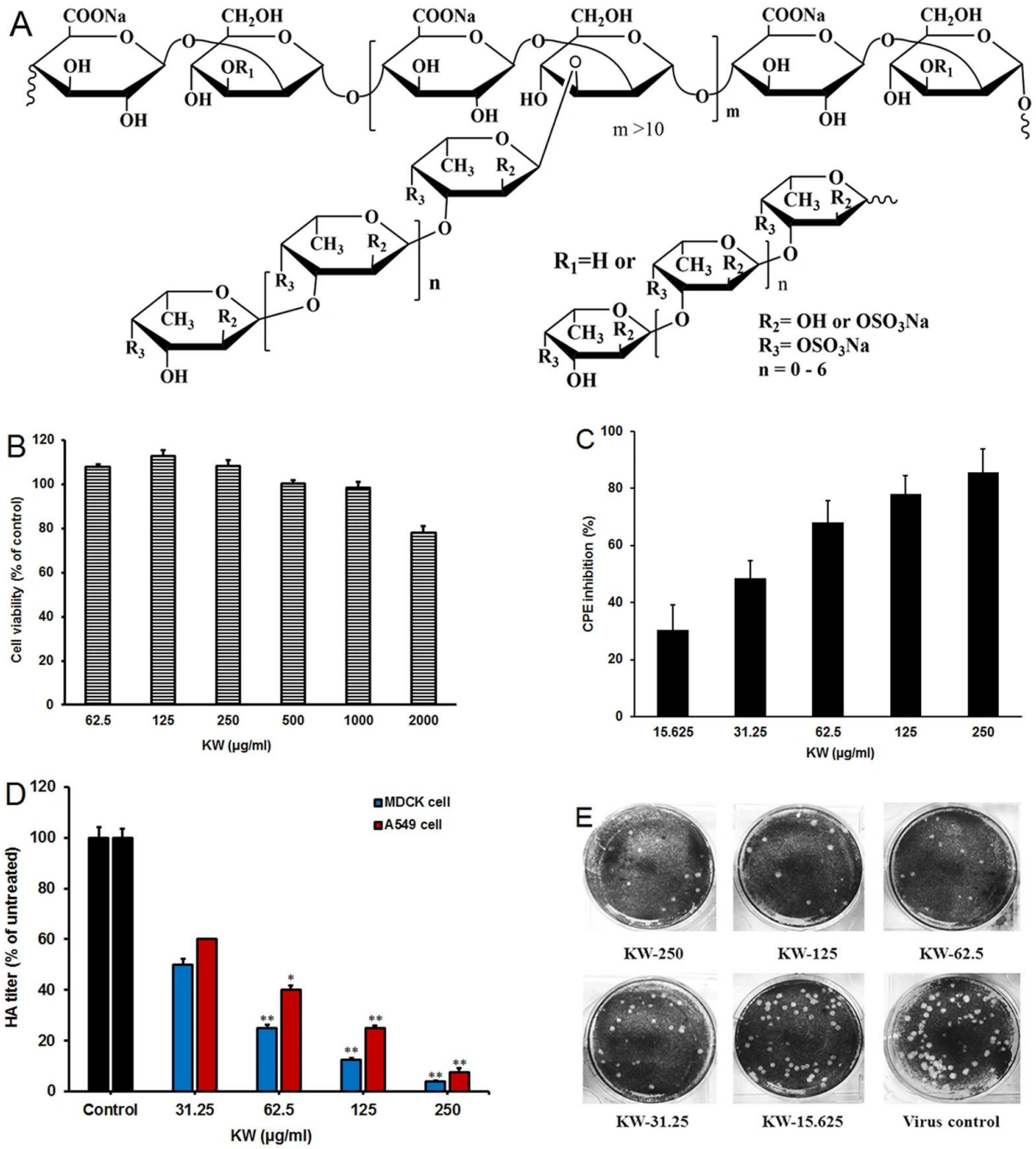

Figure 1. Fucoidan KW inhibited replication of IAV in vitro with low toxicity. (A) Schematic diagram of the chemical structure of fucoidan KW. (B) MDCK cells were exposed to different concentrations of KW in triplicate, and incubated at 37 °C for 48h. Then the cell viability was evaluated by MTT assay. (C) PR8 virus (MOI=0.1) infected MDCK cells were treated with KW at the indicated concentrations after removal of virus inoculums. (D) IAV (MOI=0.1) infected MDCK and A549 cells were treated with KW at the indicated concentrations after removal of the virus inoculum. The antiviral activity was determined by hemagglutination (HA) assay at 48h p.i. (E) Approximately 50–100 PFU/ well of PR8 was pre-incubated with different concentrations of KW for 60min at 37 °C before infection. Then the virus-KW mixture was transferred to confluent cell monolayers in 6-well plates, incubated at 37 °C for 1h and subjected to plaque assay. (Wang *et al*., 2016)

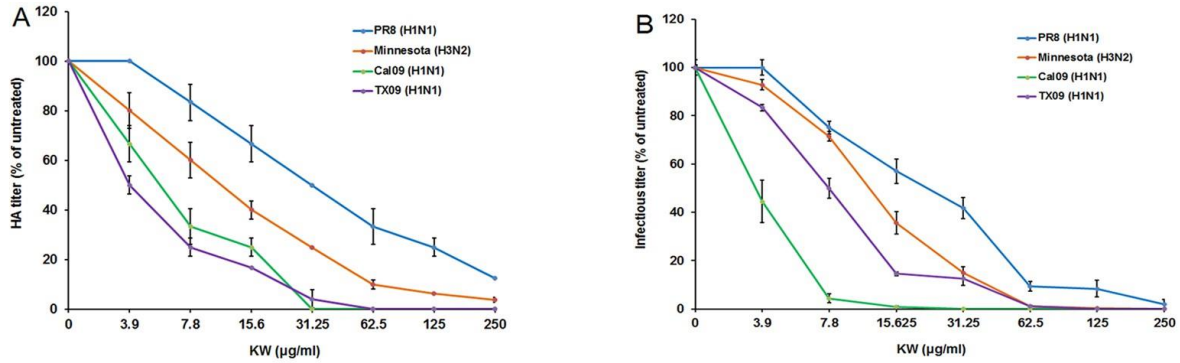

Figure 2. KW possesses broad-spectrum anti-IAV activities and low tendency of induction of viral resistance. (A) HA titers and (B) infectious virus titers from single-cycle high-moi assays performed on MDCK cells infected with PR8, Minnesota, Cal09 and TX09 and treated with the indicated concentrations of KW. Mean percentage HA titers or infectious virus titers were calculated as a percentage of HA or infectious virus titers, respectively, from untreated cells for each drug treatment condition in an experiment. Values are means ± S.D. (n=4). (Wang *et al*., 2016)

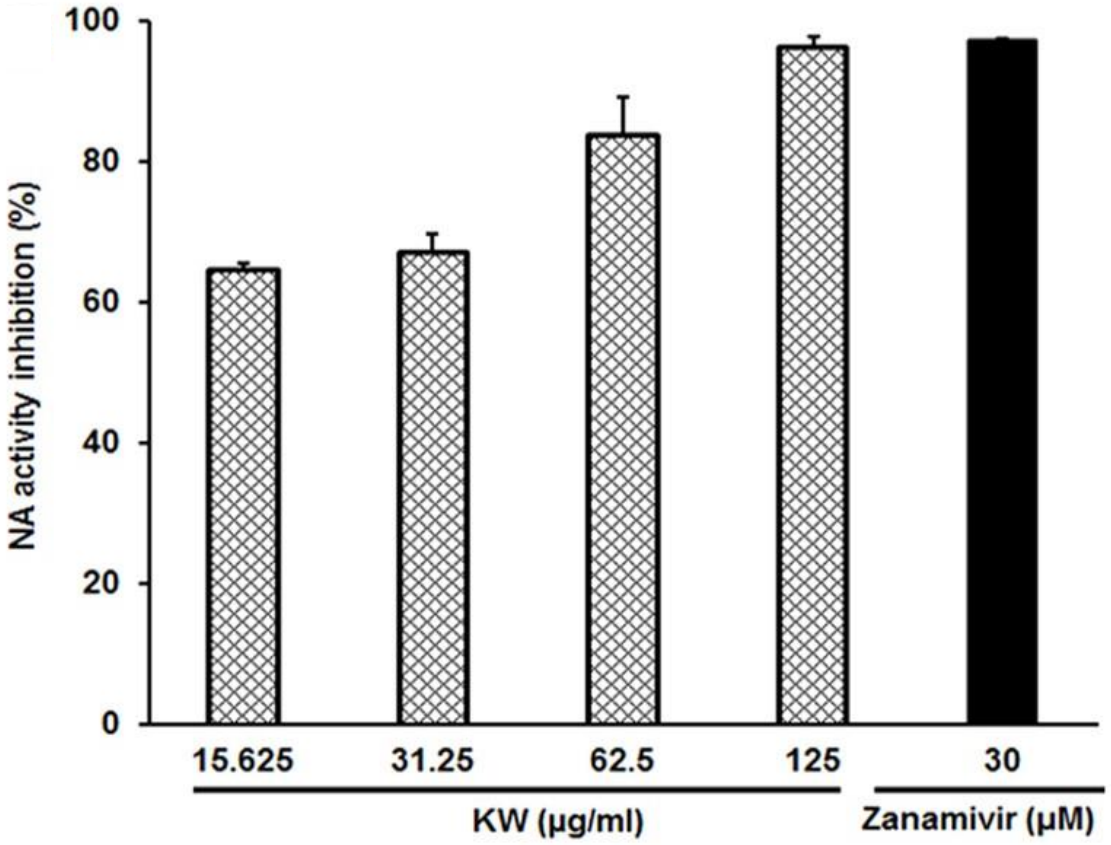

Figure 3. Influence of different treatment conditions of fucoidan on IAV infection. Inactivated PR8 virus was incubated with indicated concentrations of KW or Zanamivir (30μM), and the NA enzymatic activity was determined by a fluorescent assay. Values are means ± S.D. (n=4). (Wang *et al*., 2016)

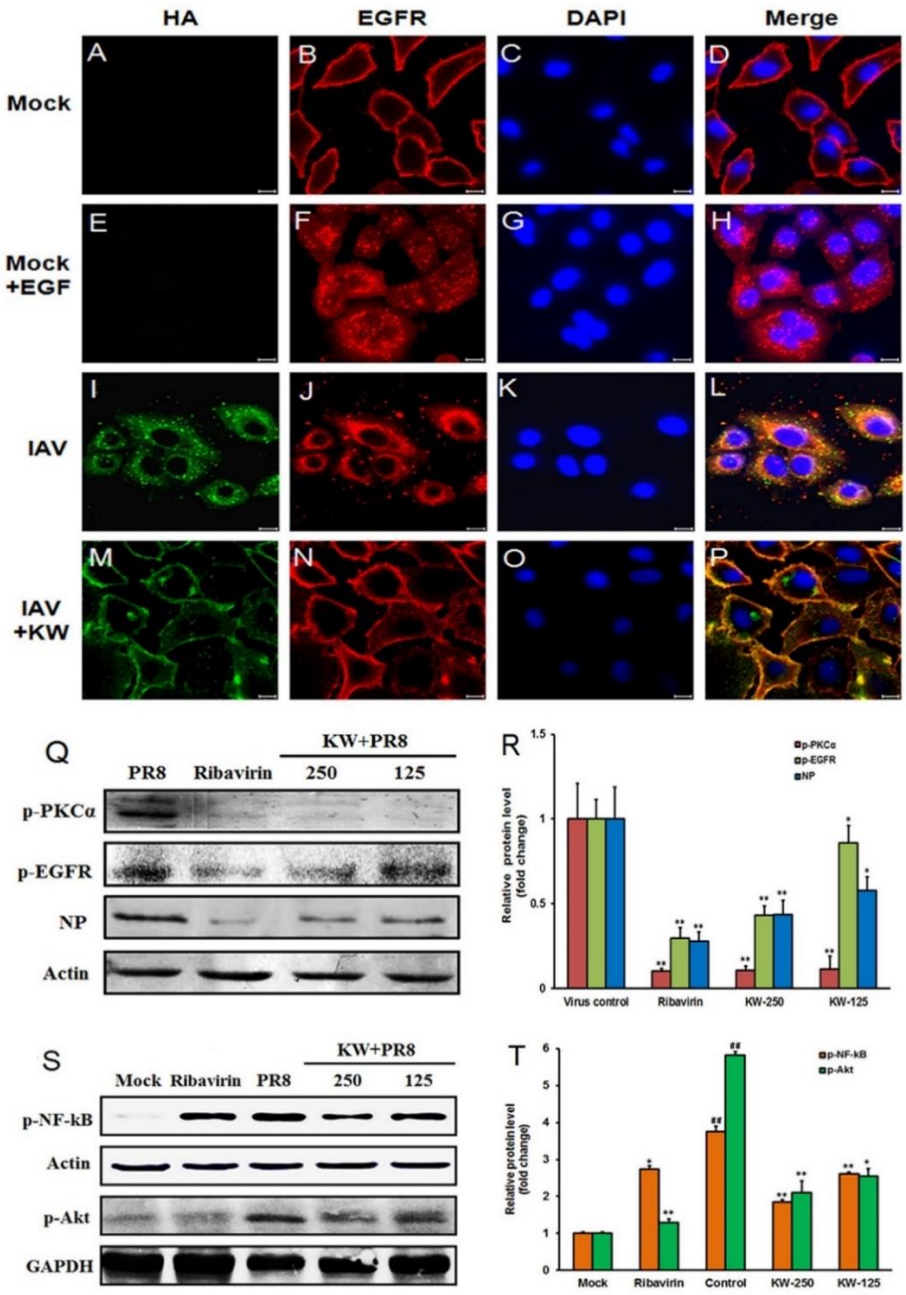

Figure 4. KW reduced IAV endocytosis through inhibition of cellular EGFR pathway. (A–P) A549 cells were infected with PR8 virus (MOI=3.0) with or without KW (250μg/ml) pretreatment at 37 °C for 1h, or were stimulated with EGF (100 ng/ml), each for 1h at 4 °C and 30min at 37 °C. An EGFR-specific rabbit antiserum and Alexa 594-conjugated goat anti-rabbit IgG as well as a HA-specific mouse antiserum and FITC-conjugated goat anti-mouse IgG were employed. (Q) IAV (MOI=1.0) infected cells were treated with or without drugs at indicated concentrations after removed virus inoculums. At 4h p.i., the expression of viral NP protein and phosphorylated PKCα and EGFR proteins were evaluated by western blot. (R) Quantification of immunoblot for the ratio of p-PKCα, p-EGFR or NP to β-actin. (S) IAV (MOI=1.0) infected cells were treated with indicated compounds for 4h, and then the phosphorylation of NF-κB and Akt proteins was evaluated by western blot. Blots were also probed for β-actin and GAPDH as loading controls. (T) Quantification of immunoblot for the ratio of p-NF-κB to actin or p-Akt to GAPDH. (Wang *et al*., 2016)

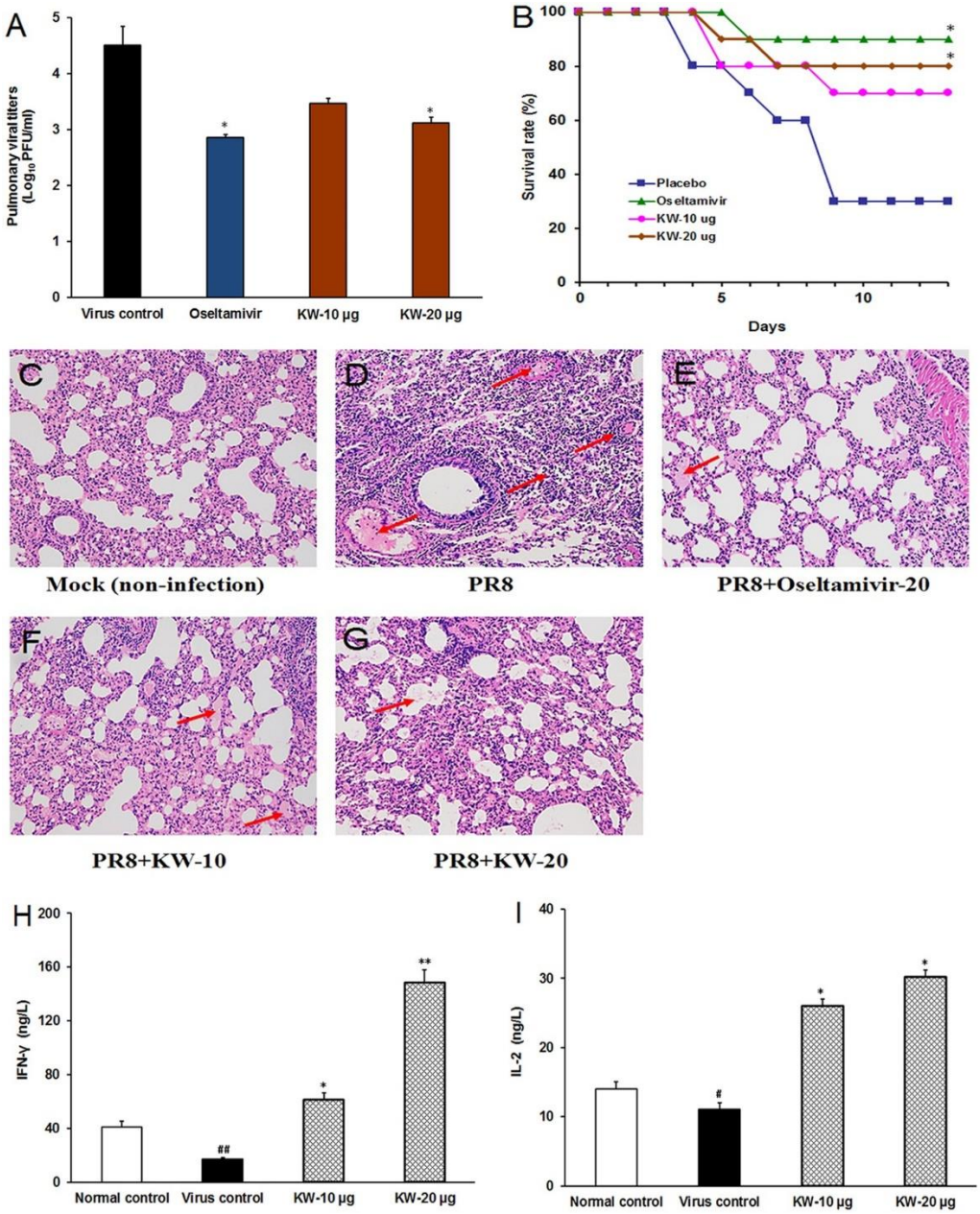

Figure 5. The anti-IAV effects of fucoidan KW in vivo. (A) Viral titers in lungs. After treatment with KW (10 or 20μg/day) or placebo (PBS) for 4 days, four mice per group were sacrificed and the pulmonary viral titers were evaluated by plaque assay on MDCK cells. (B) Survival rate. IAV infected mice were received intranasal therapy with KW (10 or 20μg/day) or placebo once daily for seven days. Results are expressed as percentage of survival, evaluated daily for 14 days. (C–G) Histopathologic analyses of lung tissues on Day 4 p.i. by HE staining (×10). The representative micrographs from each group were shown. Mock: non-infected lungs; PR8: IAV infected lungs without drugs; PR8+Oseltamivir-20: IAV infected lungs with Oseltamivir (20mg/kg/day) treatment; PR8+KW-10: IAV infected lungs with KW (10μg/day) treatment; PR8+KW-20: IAV infected lungs with KW (20μg/day) treatment. The red arrows indicate the presence of inflammatory cells in the alveolar walls and serocellular exudates in the lumen. (H,I) After treatment of KW (10 or 20μg/day) for four days, the production of interferon-γ (IFN-γ) (H) and interleukin 2 (IL-2) (I) in spleen tissues was determined by using the ELISA kits for IFN-γ and IL-2. (Wang *et al*., 2016)

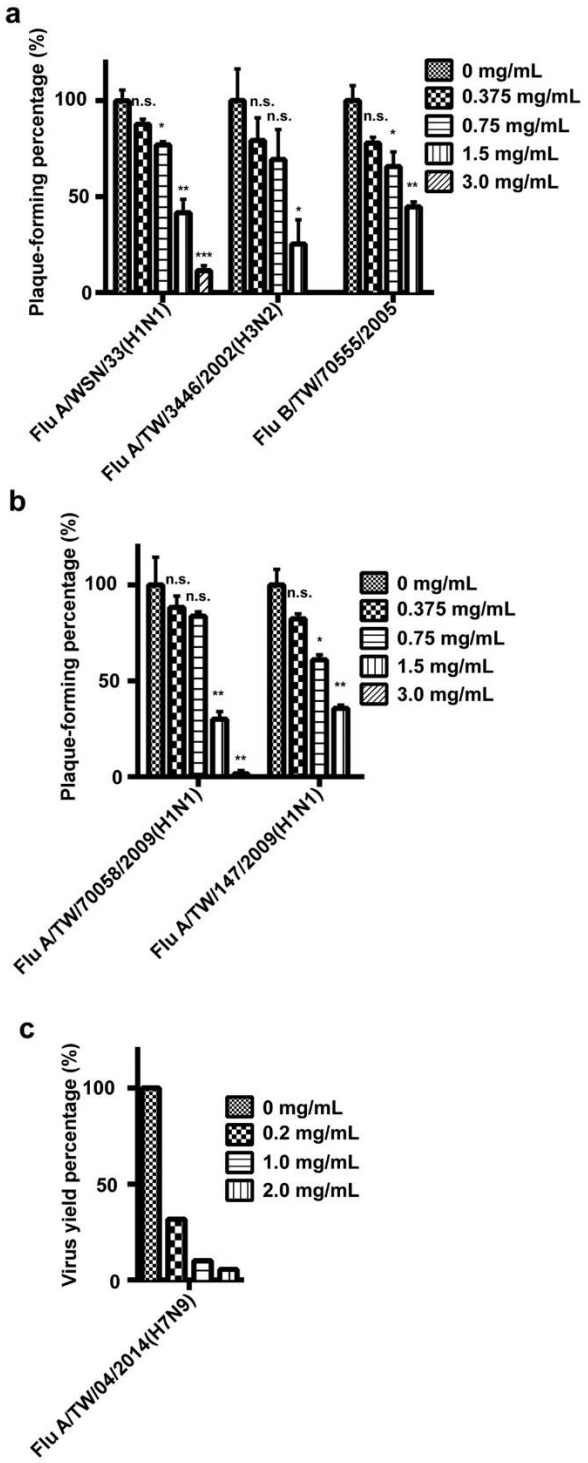

Figure 6. The cold water extract of Spirulina (Arthrospira platensis) inhibits viral replication and plaque formation in a broad range of influenza strains in vitro. (a) The influenza virus strains A/WSN/33(H1N1), A/TW/3446/02(H3N2), and B/TW/70555/05, and (b) the oseltamivir-resistant strains A/TW/70058/09(H1N1) and A/TW/147/09(H1N1), propagated on MDCK cells, all exhibited dose-dependent reductions in virus plaque formation after treatment with 0.375, 0.75, 1.5, and 3.0mg/ml of Spirulina extract. (c) The TCID50 assay showed that Spirulina extract reduced virus production of the influenza A/TW/04/2014 (H7N9) virus. (Chen *et al*., 2016)

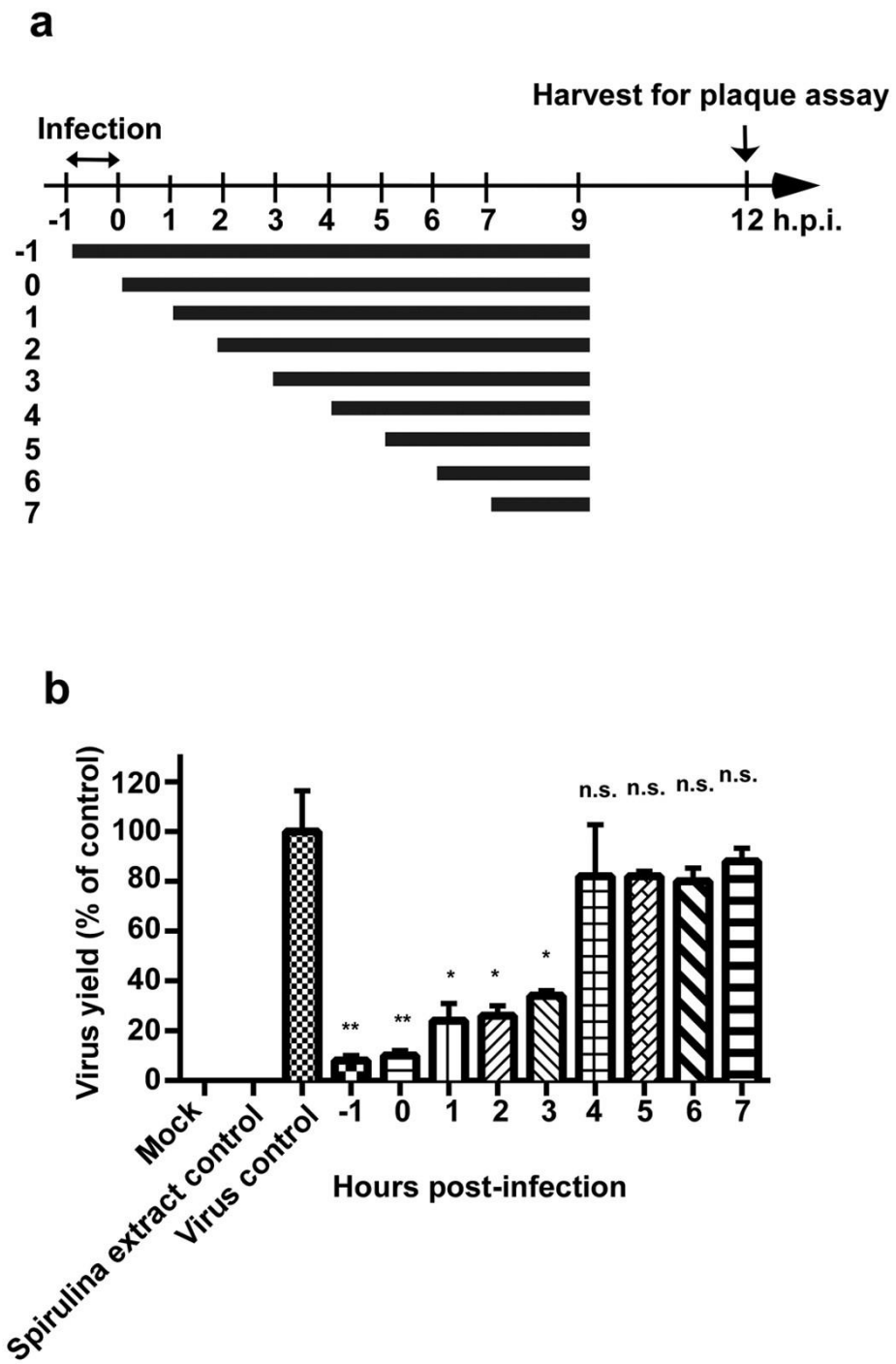

Figure 7. Spirulina extract acts to disrupt viral replication at an early stage of infection. (a) A time of addition assay was conducted, in which 2.5mg/mL of Spirulina extract was added at the indicated timepoints to MDCK cells infected with the A/WSN/33(H1N1) influenza strain. E_0 medium was overlaid on cultures at 9hours post-infection, and supernatants were harvested at 12 hours post-infection. (b) Virus yields were determined by the plaque assay, and reported as a percentage of untreated controls. Data represent the mean± SD for two independent experiments. Statistical analysis was performed with two-tailed unpaired t-test, and the statistical significance was determined to be * P≤ 0.05, **P≤ 0.01, or ***P≤ 0.001 for each treated group versus the virus control. (Chen *et al*., 2016)

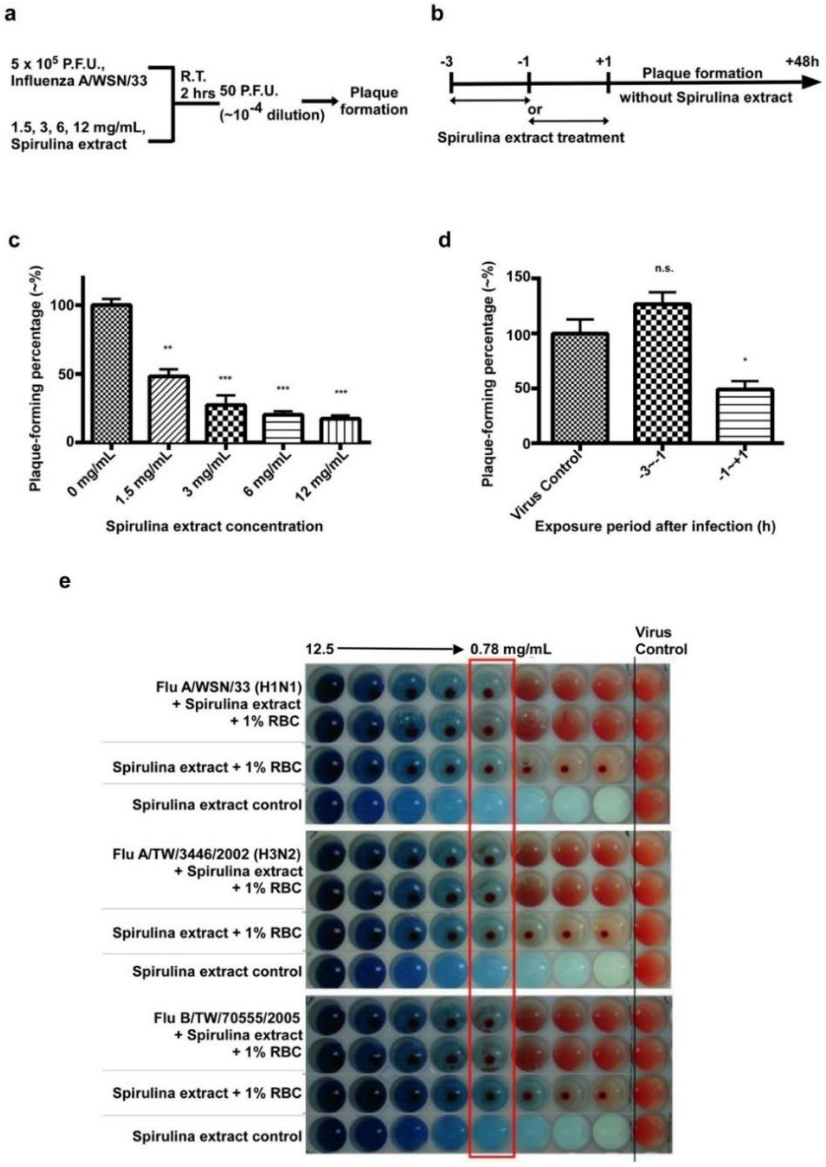

Figure 8. Spirulina extract inhibits influenza virus infection by disrupting hemagglutination. (a) 5× 10^5 PFUs of the influenza A/WSN/33(H1N1) virus was incubated with 1.5, 3, 6, or 12mg/mL of Spirulina extract at room temperature for 2 hours, after which cultures underwent a 10^4 -fold. (b) Plaque assays showed that plaque formation percentages for Spirulina extract-treated viruses were significantly lower than untreated controls. (c) Monolayer MDCK cells in 6-well plates were either treated with 3mg/mL of Spirulina extract for 2 hours, after which the culture medium was replaced with fresh medium containing no Spirulina extract (the −3~−1 group), or treated with 3mg/mL of Spirulina extract just prior to and during viral adsorption (the −1~+1 group). (d) Plaque assays showed that the plaque formation percentage of the −3~−1 group was comparable to the untreated virus control, while plaque formation was significantly inhibited in the −1~+1 group. (e) Varying concentrations of Spirulina extract were added to 96-well plates containing 1% of guinea pig RBCs and 4 HA units of influenza A/WSN/33(H1N1), A/TW/3446/02(H3N2), or B/TW/70555/05 viruses. In the virus control column at far right, no Spirulina extract was added. Results showed that concentrations of Spirulina extract above 0.78mg/mL were capable of inhibiting influenza hemagglutination. (Chen *et al*., 2016)

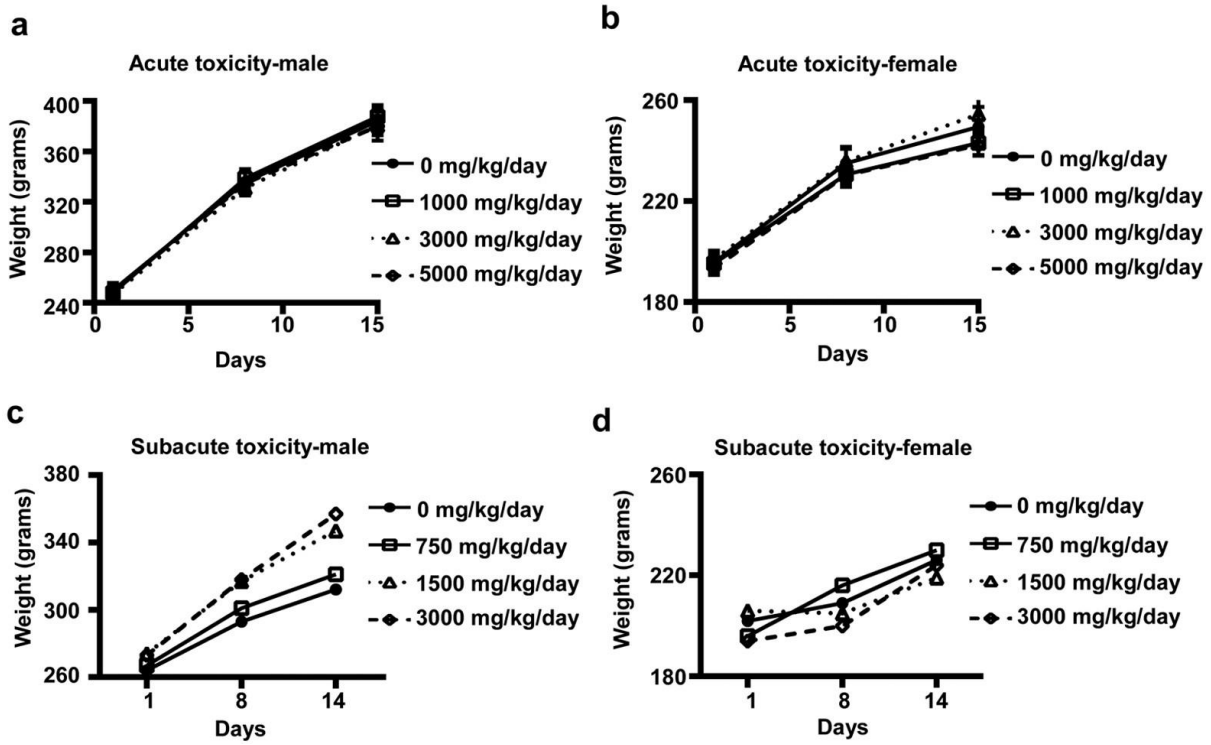

Figure 9. Short-term acute and subacute oral toxicity for the Spirulina extract. (a) 14-day acute oral toxicity study was conducted in Sprague-Dawley rats given 0, 1,000, 3,000, or 5,000mg/kg of Spirulina extract. Body weight changes were tracked for (a) male and (b) female rats during the study period, and no significant changes were observed. (a) 14-day subacute study, in which Sprague-Dawley rats were subjected to repeated dosing with 750, 1,500, or 3,000mg/kg/day of Spirulina extract for 14 consecutive days, was also conducted. Body weight changes for (c) male and (d) female rats were measured on Day 1, Day 8, and Day 14. All data are presented as mean ± SD (N= 10). (Chen *et al*., 2016)

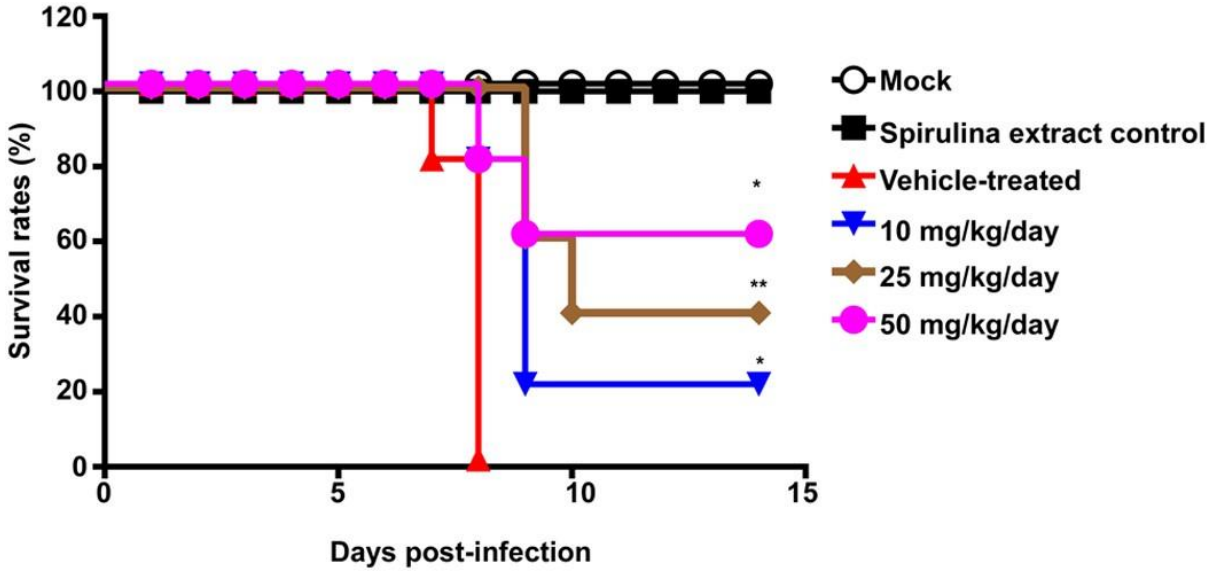

Figure 10. Spirulina extract improves survival rates in influenza-infected mice. Six-week-old female BALB/c mice were inoculated with 2× 10^4 PFUs of the influenza A/WSN/33(H1N1) virus, after receiving Spirulina extract 4 hours prior to infection by oral gavage. Spirulina extract was again administered at 6 hours post-infection and twice daily thereafter for 4 days, at respective daily doses of 10, 25, or 50mg/kg. Survival rates were improved in mice treated with Spirulina extract, as compared to vehicle-treated controls that did not receive Spirulina extract. Statistical analyses of the survival curves were performed using the Log-rank (MantelCox) test compared each Spirulina extract treated group to the vehicle-treated group (n= 5 mice per group). *P< 0.05, **P< 0.01. (Chen *et al*., 2016)

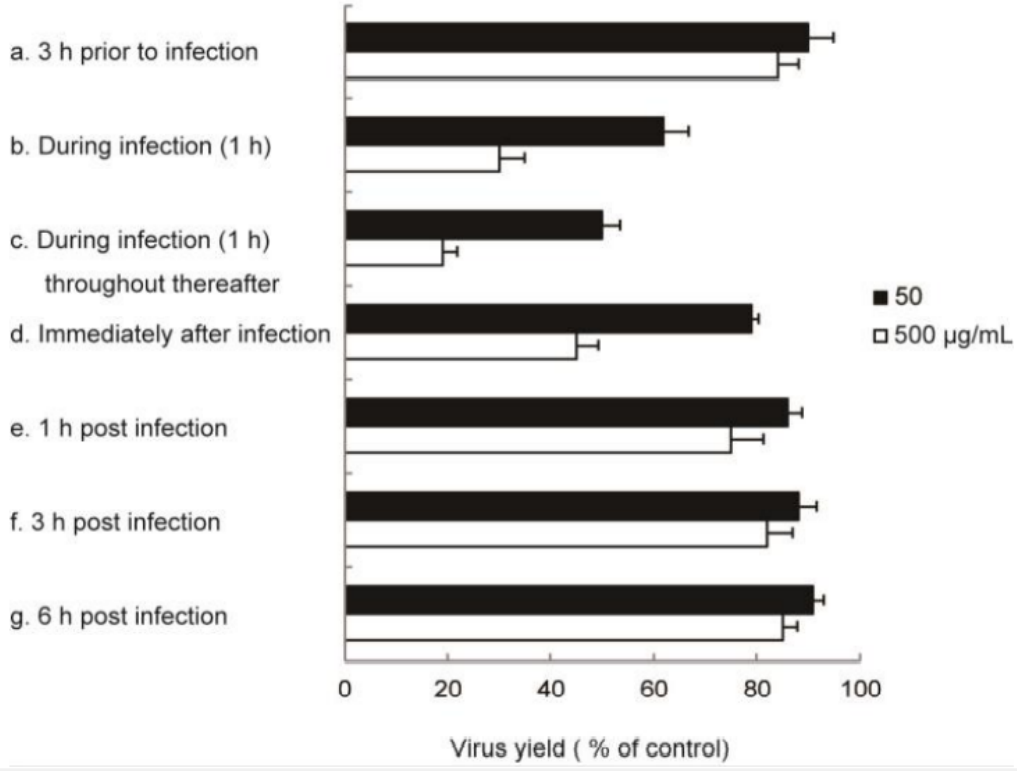

Figure 11. Effects of time-of-addition of RS on IFV replication. RS at a concentration of 50 µg/mL (closed bar) or 500 µg/mL (open bar) was added to the culture medium 3 h prior to virus infection (a), during infection for 1 h (b), during infection for 1 h, and throughout the subsequent incubation (c), immediately after infection (d), at 1 h post-infection (p.i.) (e), at 3 h p.i. (f), or at 6 h p.i. (g). Each value is expressed as the mean ± SD from independent duplicate assays. (Masahiro *et al*., 2020)

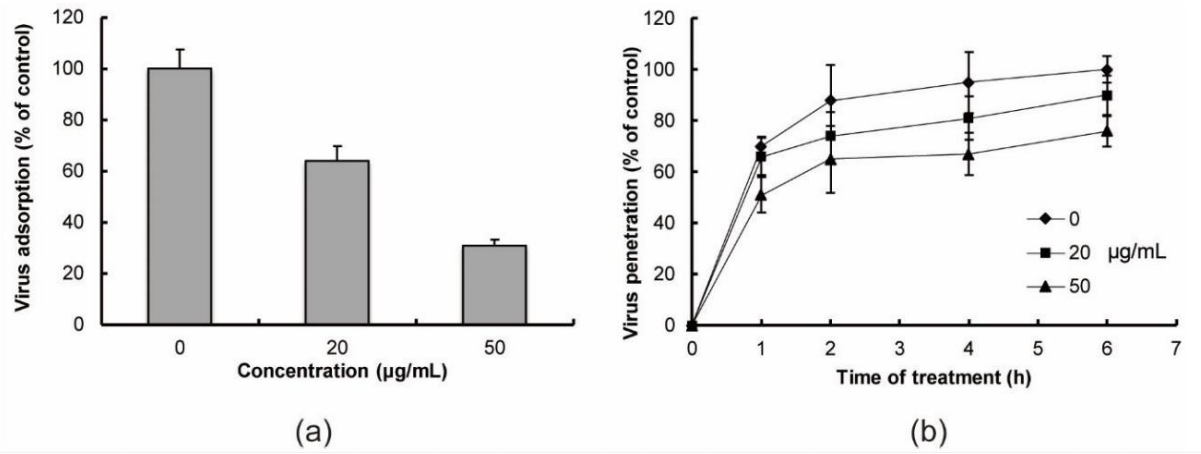

Figure 12. Effects of RS on virus adsorption and penetration. (a) For viral adsorption inhibition assays, Madin–Darby canine kidney (MDCK) cell suspensions were infected with IFV (1 PFU/cell) in the absence or presence of RS (20 or 50 μg/mL) at 4 °C for 1 h prior to plaque assays. (b) In viral penetration assays, cell monolayers were infected at 4 °C with IFV in the absence of RS, and then shifted to 37 °C to allow virus penetration in the presence of RS for indicated time. Virus titers were determined by the plaque assay. Closed diamond, no drug control; closed square, 20 μg/mL RS; and closed triangle, 50 μg/mL RS. The number of plaques in the control 6 h after temperature shift was assigned as 100%. Each value is the mean ± S.D. from triplicate assays. (Masahiro *et al*., 2020)

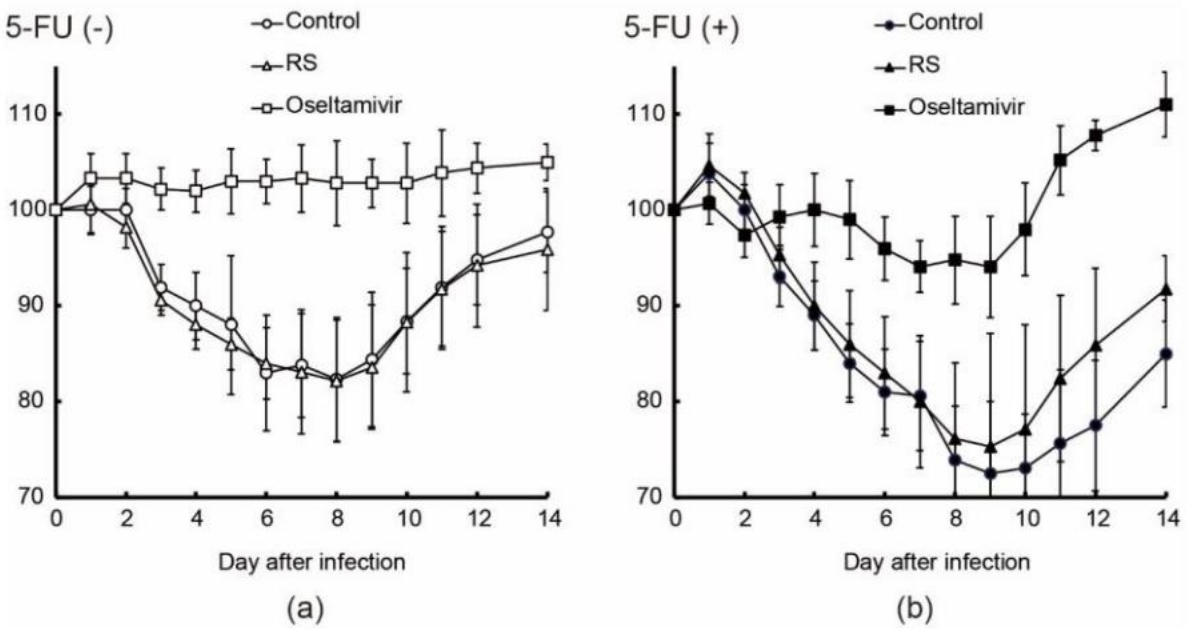

Figure 13. Effects of RS and oseltamivir on mouse body weight change. IFV-infected mice were subcutaneously injected with or without 5-FU, and they were orally administered with water (open and closed circles; control), 5 mg RS (open and closed triangles), or 0.5 mg oseltamivir (open and closed squares) per day. Changes in body weight in 5-FU (-) mice (a) and 5-FU (+) mice (b) are shown (n = 6–11). Bodyweight on the day of viral infection (0 d) was taken as 100%. (Masahiro *et al*., 2020)

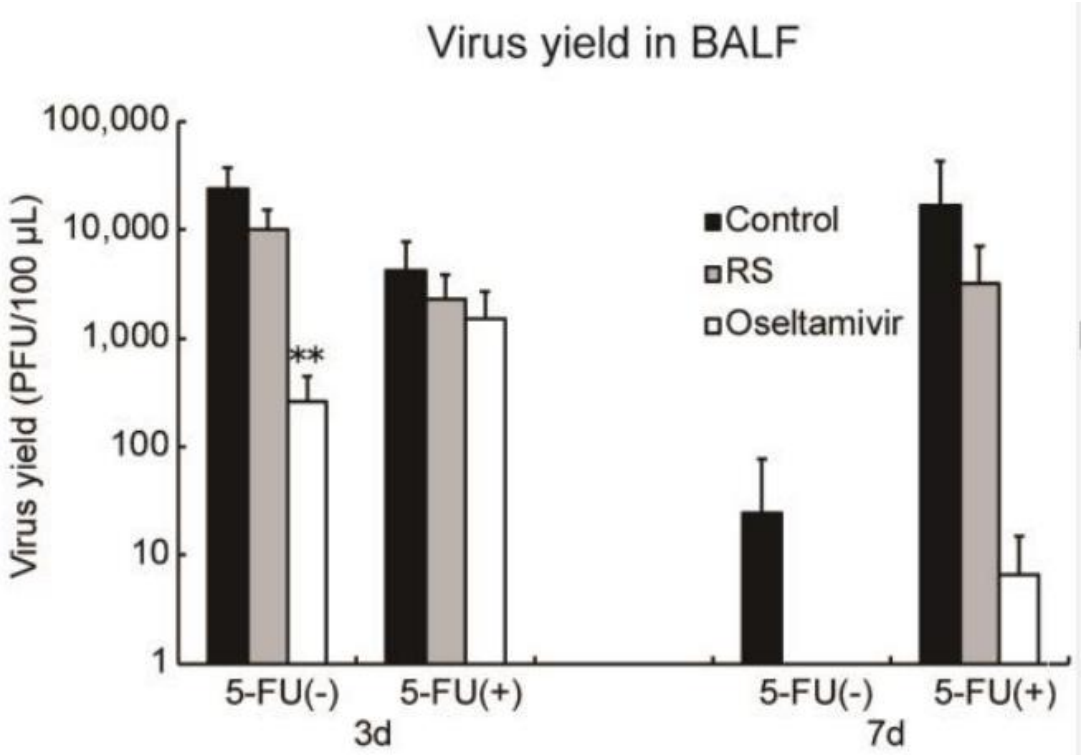

Figure 14. Effects of RS and oseltamivir on virus production in lung and bronchoalveolar lavage fluid (BALF) samples of mice taken 3 or 7 days after IFV infection. IFV-infected mice were subcutaneously injected with or without 5-fluorouracil (5-FU) and were orally administered with water (closed columns, control), 5 mg RS (gray columns), and 0.5 mg oseltamivir (open columns). Virus yields in the (a) lung and (b) BALF samples are shown. Each value represents the mean ± S.D (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group. ## $p < 0.01$ against the oseltamivir-administered FU (-) group. (Masahiro *et al*., 2020)

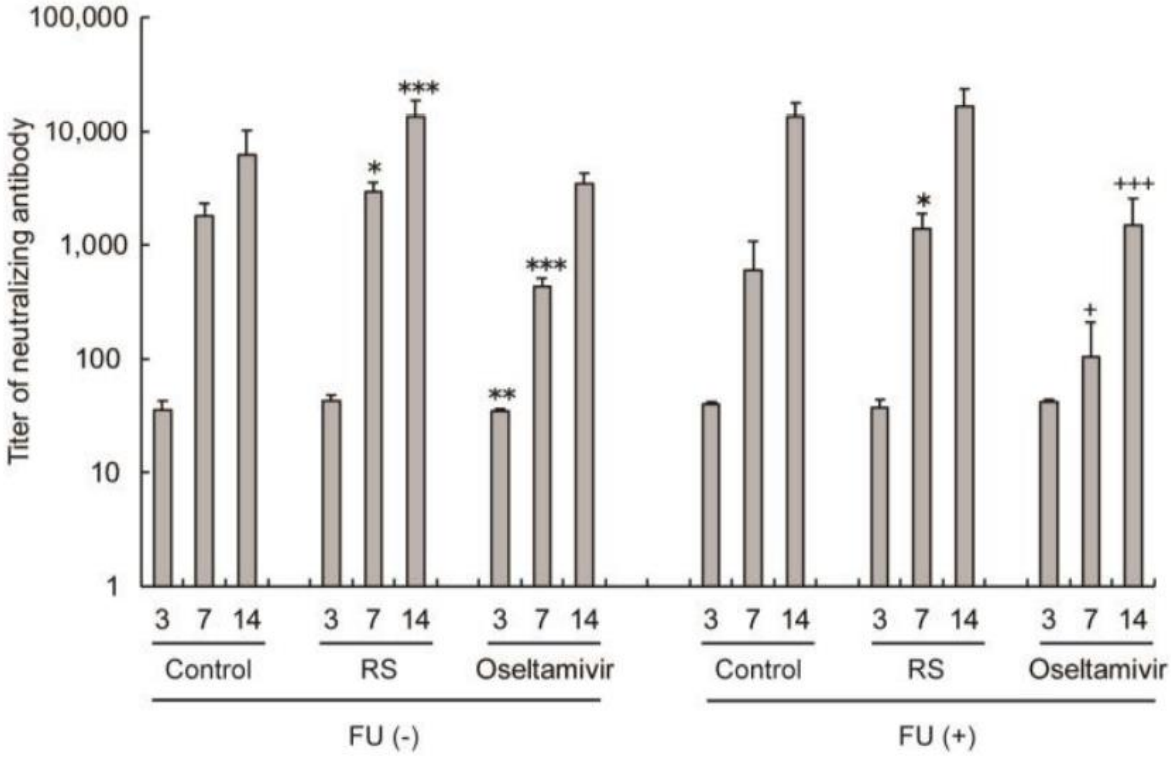

Figure 15. Effects of RS and oseltamivir on the production of neutralizing antibodies against IFV in serum samples of mice taken at 3, 7, or 14 days after IFV infection. IFV-infected mice were subcutaneously injected with or without 5-FU and were orally administered with water (control), 5 mg of RS, and 0.5 of mg oseltamivir. Titers of neutralizing antibody in the serum are shown. Each value represents the mean ± S.D (days 3 and 7, n = 5; day 14, n = 6–11). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group. + $p < 0.05$, +++ $p < 0.001$ versus the control or RS-administered group. (Masahiro *et al*., 2020)

Table 1. Hematology results in rats (Mean ± SD, N=10)

Parameters	Sex	Dose (mg/kg/day)			
		0	750	1500	3000
WBC (10^3/μL)	Male	11.058 ± 3.260	11.769 ± 3.089	14.721 ± 3.362	13.434 ± 4.067
	Female	11.158 ± 3.107	11.104 ± 3.626	11.387 ± 2.467	11.280 ± 4.141
RBC (10^6/μL)	Male	7.867 ± 0.238	8.066 ± 0.456	7.889 ± 0.375	7.809 ± 0.236
	Female	8.447 ± 0.488	8.287 ± 0.585	8.558 ± 0.555	8.392 ± 0.517
Hb (g/dL)	Male	14.83 ± 0.52	15.22 ± 0.94	15.19 ± 0.57	15.00 ± 0.57
	Female	15.64 ± 0.91	15.47 ± 1.19	15.98 ± 0.78	15.69 ± 0.83
Ht (%)	Male	46.98 ± 1.41	48.19 ± 2.82	47.80 ± 1.75	47.14 ± 1.83
	Female	48.35 ± 2.94	47.64 ± 3.34	48.93 ± 2.34	48.23 ± 2.51
MCV (fl)	Male	59.66 ± 0.88	59.76 ± 1.13	60.58 ± 1.01	60.37 ± 1.40
	Female	57.24 ± 1.60	57.52 ± 0.88	57.24 ± 1.75	57.52 ± 1.63
MCH (pg)	Male	18.86 ± 0.40	18.87 ± 0.41	19.26 ± 0.27	19.22 ± 0.47
	Female	18.49 ± 0.50	18.68 ± 0.33	18.67 ± 0.68	18.71 ± 0.46
MCHC (%)	Male	31.59 ± 0.46	31.58 ± 0.27	31.81 ± 0.41	31.81 ± 0.38
	Female	32.32 ± 0.37	32.45 ± 0.46	32.63 ± 0.64	32.53 ± 0.46
Platelet (10^3/μL)	Male	1134.0 ± 155.0	1152.4 ± 156.4	1088.8 ± 168.8	1150.2 ± 147.8
	Female	1219.0 ± 86.0	1197.0 ± 164.8	1244.3 ± 104.5	1209.2 ± 106.5

*: $P < 0.05$

(Chen *et al*., 2016)

Table 2. Effects of RS and oseltamivir on mortality.

Sample	FU-Treat	Survived/Challenged	Mortality	Day of Death
Control	-	11/11	0%	
RS	-	11/11	0%	
Oseltamivir	-	10/10	0%	
Control	+	9/11	18%	9, 10 d
RS	+	10/10	0%	
Oseltamivir	+	6/6	0%	

(Masahiro *et al*., 2020)