"알찬 대학 따뜻한 동행"

TOP SECRET

전북대학교

수신자 (주)LSK화인텍스 대표이사
(경유)
제목 노하우 기술이전 결과보고서 송부

1. 귀 사의 무궁한 발전을 기원합니다.

2. 귀 사와 전북대학교 산학협력단이 2020년 6월 22일에 체결한 노하우 기술이전 계약과 관련하여 해당기술 이전을 위한 결과보고서를 붙임과 같이 송부드리며, 아울러 계약서 제6조(비밀보장) 제1항에 의거하여 관련정보의 비밀유지에 만전을 기해 주시기 바랍니다.

가. 계약기술 : 코로나 바이러스의 (주)LSK화인텍스 COPPER 원단소재에 대한 항 바이러스 실험 및 연구
나. 발 명 자 : 전북대학교 수의과대학 교수 이존화
다. 이전기업 : ㈜LSK화인텍스
라. 계 약 일 : 2020년 6월 22일

붙임 1. 기술이전 계약서 1부
2. 결과보고서 1부. 끝.

전북대학교 산학협력단장

실무관	이희상	실무팀장	임용환	부장	이현승	과장	박성일
센터장	전결 2020.08.21. 최남석						

협조자

시 행 Rn8D전략센터-2816 (2020.08.21.) 접 수 ( )

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Assessment of COVID-19 virucidal activity of 3D layered structure with copper-base Copperline face mask of LSK FINETEX

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COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a beta-coronavirus, similar to severe acute respiratory syndrome coronavirus (SARS CoV). The pandemic of COVID-19 is growing, and a shortage of masks and respirators has been reported globally. Prevention of infection with airborne pathogens (viruses, bacteria) can be facilitated through the use of disposable face masks. Development of a biocidal mask (and in general, all protective personal equipment (PPE)) capable of rendering the pathogens that come into contact with them non-infectious, may significantly reduce pathogen transmission and contamination of the wearers themselves and the environment. Copper has potent biocidal properties. For example, copper inactivates bacteriophages [1], bronchitis virus [2], poliovirus [3], herpes simplex virus [4], human immunodeficiency virus (HIV) and influenza viruses [5,6]. In the present study, we will evaluate the anti-corona biocidal properties of copper that have incorporated into the fabric of the mask acts as a barrier to the transmission of COVID-19 coronavirus.

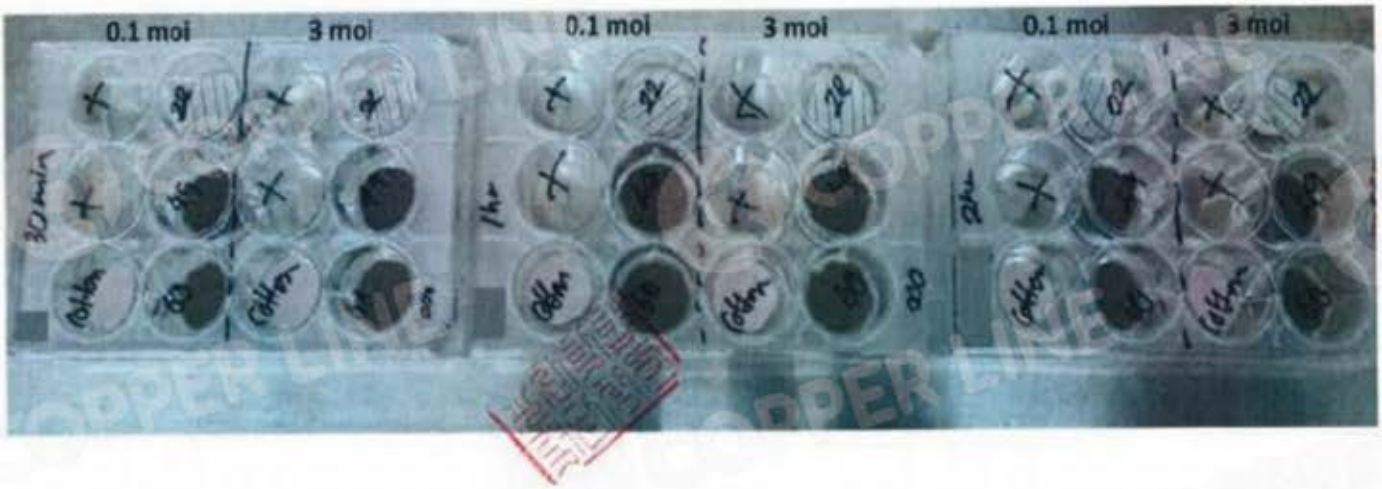
Methodology

Virus and cells

Vero E6 cells were obtained from the American Type Culture Collection (ATCC CRL-1586) and maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1× antibiotic-antimycotic solution (Gibco). SARS-CoV-2 (BetaCoV/Korea/KCDC 03/2020) was provided by the Korea Centers for Disease Control and Prevention (KCDC) and was propagated in Vero E6 cells. All experiments using SARS-CoV-2 were performed at Chonbuk National University Zoonotic Center, using enhanced biosafety level 3 (BSL3) containment procedures in laboratories approved for use by the KCDC.

Cytopathic effects (CPE)

5×10^4 cells/well Vero E6 cells were seeded into 12-well plates in 10% DMEM growth medium and cultured overnight at 37°C in 5% CO_2. Before the experiment mask materials were autoclaved and dried. Then face mask materials will be placed on a new 12 well plate tissue culture plate.



For the viral infections, plates were transferred into the BSL3 containment facility and the membranes will be incubated with SARS-CoV2 at different time points (30min, 1hr, and 2hr) with a multiplicity of infection (moi) of 0.1, (Pfu/ml = 10,000/ml).

Calculation of infection dose (0.1 moi);

moi; multiplicity of infection

Virus titer of the original stock: 1×10^6/ml

Cell number : 1×10^5 cells/well

Pfu/ml = 0.7× virus titer

= $0.7\times 1\times10^6$

= 700,000/ml

$$\text{moi of original virus stock} = \frac{\text{Pfu/ml}}{\text{Cell number}}$$

= $700{,}000/1 \times 10^5$

= 7 moi

Media of plates containing cells will be removed and washed with 1X PBS meantime after each incubation time, membranes will be washed with 500µl of DMEM and added to the cell culture plates accordingly. Then the cell culture plates will be incubated at 37°C in 5% CO2 incubator for 1 hour.

After 1 hour incubation infection media was removed and 1ml of DMEM containing 2% FBS was added and cultured at 37°C in 5% CO2 incubator for 3 days. After 3rd day of post-infection the cytopathic effects (CPE) were observed under light microscopy.

Immunofluorescence assay (IFA)

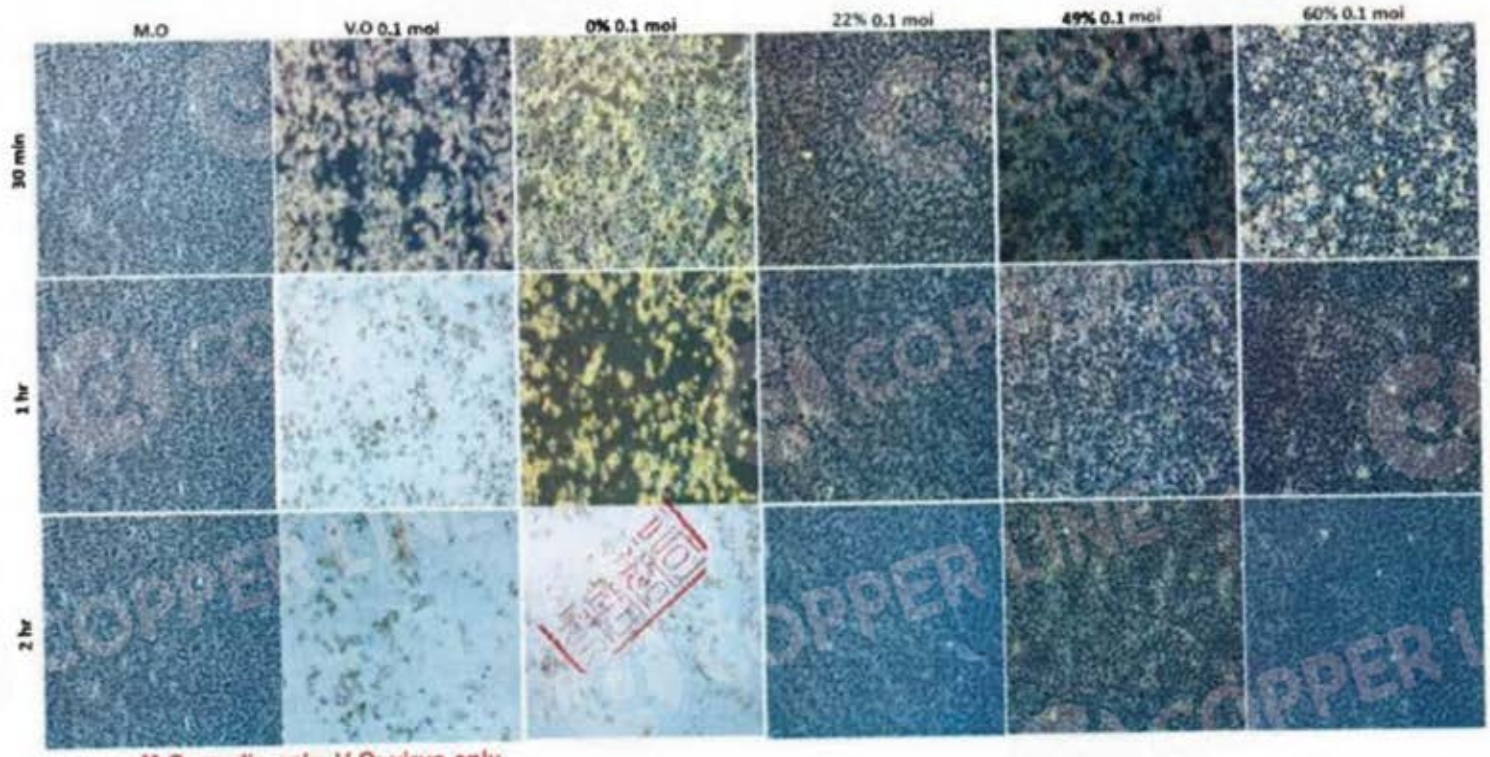
To more intuitively observe the antiviral effect of copper on SARS-CoV2, we performed an immunofluorescence assay. The monolayer of Vero E6 cells infected with 0.1 moi of SARS-CoV2 was exposed to different copper percentages and then fixed in 80% cold acetone for 30 min. Next, the cells were washed 3 times with PBS containing. Then, the primary antibody (SARS-CoV2 S protein, Sino Biological) diluted at a ratio of 1:1000 with 5% BSA was added, and the cells were incubated overnight at 4 °C. The supernatant was discarded, the cells were washed 3 times with PBS, and 1:5000 diluted FITC-labelled Alexa fluor anti-rabbit secondary antibody was added to the cells in the dark and incubated at 37 °C for 1 h. The fluorescence was observed under an inverted Leica fluorescence microscope (Leica, Germany).

Quantitative Real-Time PCR

Virus-containing supernatants were collected from the cells infected with virus (0.1 moi) with SARS CoV2 at 30min, 1 hour, and 2 hours post-infection. Total RNA was isolated using viral takara Viral DNA/RNA Extraction Kit and used for cDNA synthesis using elpis Reverse transcriptase (elpis Biotech, Daejeon, Korea) according to the manufacturer's instruction. Real-time PCR using applied biosystems (Massachusetts, USA) was performed by subjecting the reaction mixtures to initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 20 sec, 54°C for 20 sec, and 72°C for 30 sec. The primer sequences specific for the Nucleocapsid gene of the SARS CoV2 virus are used for PCR (forward CACATTGGCACCCGCAATC, reverse GAGGAACGAGAAGAGGCTTG).

Results

Cytopathic Effect (CPE) Assay

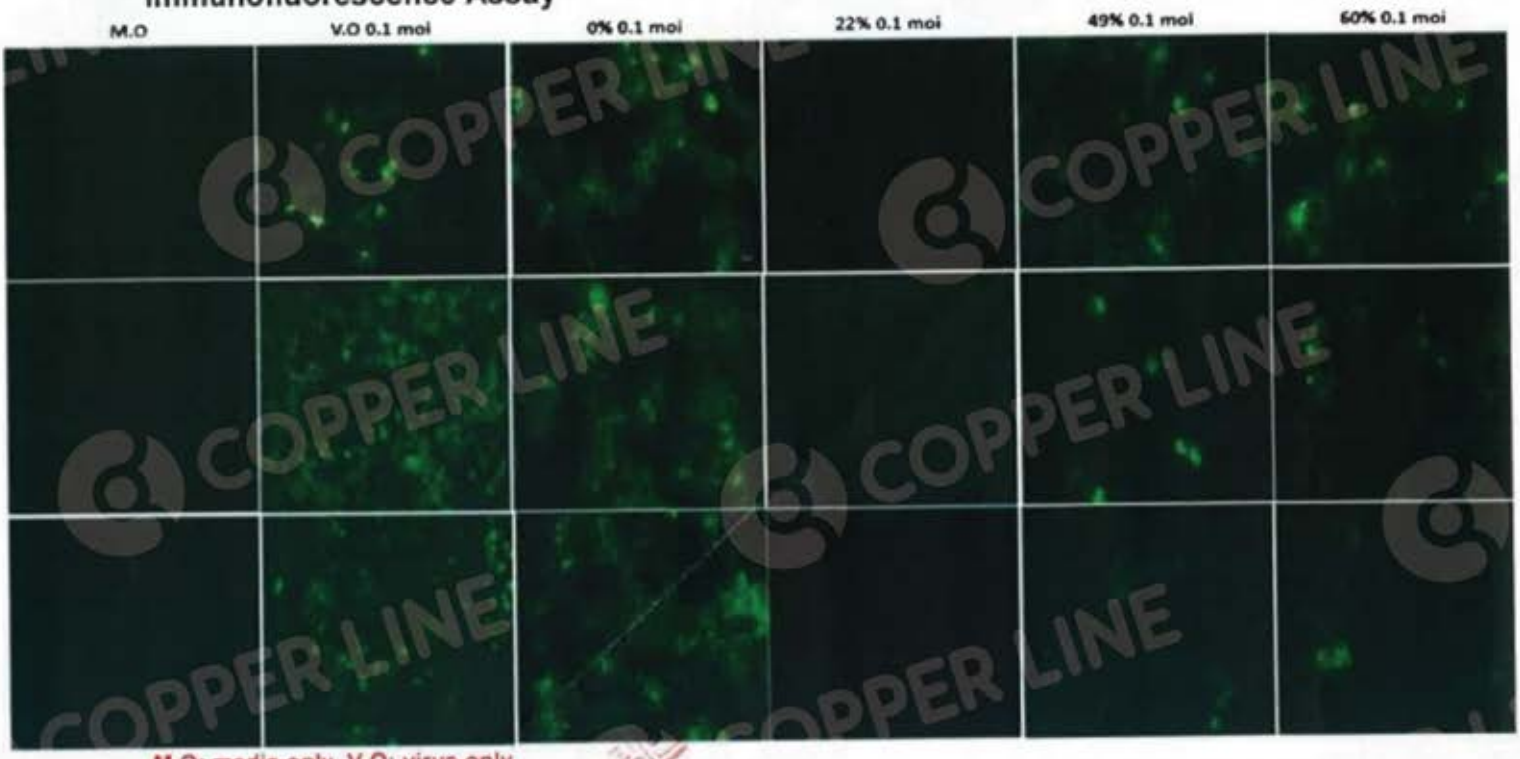


M.O: media only, V.O: virus only

The mask materials with different copper percentages were screened for their antiviral activities by using CPE inhibition assay. Vero E6 cells were infected at 0.1moi (10,000 pfu/ml) and incubated for 30min, 1 hour, and 2 hours. As shown in figure mask with different copper percentages showed significant inhibition of the cytopathic effect induced by the SARS-CoV2.

Non infected VERO E6 cell monolayer after 72 hours, showed no CPE. Mask material with 0% of copper was inactive against SARS-CoV2 at each different infection time points. **After 30 min of infection, the 3D textured with copper-base mask materials with 22% of copper had nearly 100% and the 49% and 60% of copper coated general mask materials had approximately 80% of inhibitory effect against SARS-CoV2.** After 1 hour and 2 hours of infection, compared to each other no compound appeared to be significantly superior in activity to another among the copper percentages tested.

Immunofluorescence Assay

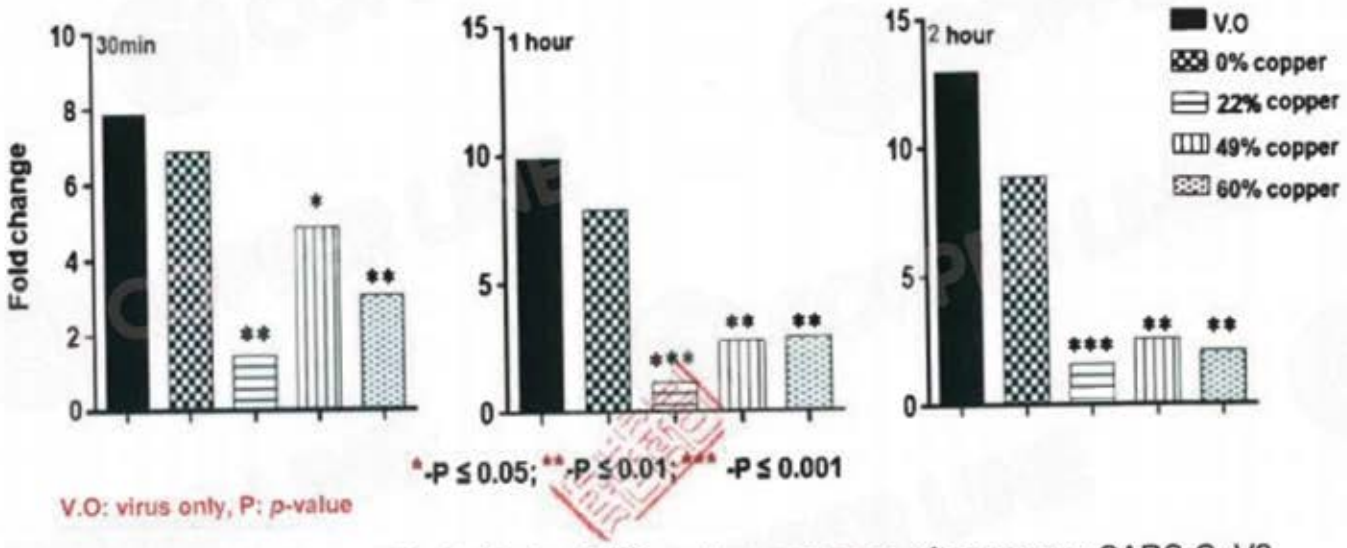


M.O: media only, V.O: virus only

To study the inhibitory impact of copper on the expression of SARS-CoV2 virus S protein on the Vero-E6 cells surface, an indirect immunofluorescence assay (IFA) was performed including both negative and positive controls.

According to the results, a substantial decrease in fluorescence emission intensity in SARS CoV2-infected cells treated with different copper percentages of 22, 49, and 60 compared to an intense green fluorescence signal that was observed 0% copper treatment group. There was almost no fluorescence signal at cells treated with **the 3D textured with copper-base mask materials with 22% of copper** to that of the control group.

Quantitative real time RT-PCR (qRT-PCR)



To evaluate the antiviral effects of different percentages of copper on SARS-CoV2, we examined RNA levels of SARS-CoV2 in Vero E6 cells after incubation SARS-CoV2 on different percentages of copper-based mask materials.

After 30 mins of infection, in the presence of 22%, 49%, and 60% copper, the SARS-CoV2 RNA replication levels were reduced by 2.8 (64.4%),1.4 (35.9%), and 2.2 (55.6%)-fold, respectively. Furthermore, after 1hour of post-infection the mRNA levels were reduced by 6.8 (92.8%), 2.8 (76.8%), and 2.7 (75.96%) fold in 22%, 49%, and 60% copper treated cells. A more pronounced decline in the relative expression of the SARS-CoV2 N gene was found when the virus was incubated for 2 hours by 5.6 (90.5%), 3.4 (80.8%) and 4.2 (85.3%) folds respectively.

Conclusion

A variety of respiratory pathogenic agents such as influenza, SARS-CoV, MERS-CoV have been exposed to a variety of copper forms in several cultivating media (MDCK, Vero, etc.) having similar results and same conclusion: Copper is capable to inhibit, inactivate, reduce and irreversibly destroy coronavirus, influenza virus, and other pathogenic agents. A recent study has evaluated and compared SARS-CoV-1 and SARS-

CoV-2 stability and decay rates in copper, no viable virus was observed after 8 hours and after 4 hours of SARS-CoV-1 and SARS-CoV-2 respectively.

The described data appears to support the use of copper in different percentages to actively inactivate SARS-CoV2 viruses and it seems to be effective, limiting environmental contamination and a low-cost strategy in reducing transmission of infectious diseases such as the SARS-CoV. It appears that superoxide and hydroxyl radical generation may be important in the inactivation of coronaviruses on copper but that inactivation is primarily due to the direct effect of copper ions. However, the described data support the incorporation of copper in different percentages (22%, 49%, and 60%) increases cell viability by reducing the viral load. The Vero E6 cells treated with copper percentages of 22%, 49%, and 60% showed cell activity greater than 80%, which was similar to that previously reported for the cytotoxicity of soluble copper [7]. After treatment with different concentrations of copper, the viral titer and RNA expression levels of SARS-CoV2 in cells were significantly reduced from those in the control cells.

Interestingly, **the 3D textured with copper-base mask materials with 22% of copper** had a higher capacity to readily kill the virions that remain in the mask. The reason for this major significance may be due to its increased surface area, and hence improve their microbicidal action via the interaction of copper ions with the virions that are entrapped in the mask or that come into contact with the surface of the copper impregnated outer surfaces of the masks.

Nevertheless, more research would be beneficial to support its usage of copper along with effective formulation against Coronaviruses.

References

[1] Yahya MT, Gerba CP, Straub TM, Margolin AB. Inactivation of bacteriophage MS-2 and poliovirus in copper, galvanized and plastic domestic water pipes. Int J Environ Health Res 1991. doi:10.1080/09603129109356707.

[2] Jordan FT, Nassar TJ. The influence of copper on the survival of infectious bronchitis vaccine virus in water. Vet Rec 1971. doi:10.1136/vr.89.23.609.

[3] Sagripanti JL, Routson LB, Lytle CD. Virus inactivation by copper or iron ions alone and in the presence of peroxide. Appl Environ Microbiol 1993. doi:10.1128/aem.59.12.4374-4376.1993.

[4] Sagripanti JL, Routson LB, Bonifacino AC, Lytle CD. Mechanism of copper-mediated inactivation of herpes simplex virus. Antimicrob Agents Chemother 1997. doi:10.1128/aac.41.4.812.

[5] Sagripanti JL, Lightfoote MM. Cupric and ferric ions inactivate HIV. AIDS Res Hum Retroviruses 1996. doi:10.1089/aid.1996.12.333.

[6] Horie M, Ogawa H, Yoshida Y, Yamada K, Hara A, Ozawa K, et al. Inactivation and morphological changes of avian influenza virus by copper ions. Arch Virol 2008. doi:10.1007/s00705-008-0154-2.

[7] Borkow G, Zhou SS, Page T, Gabbay J. A novel anti-influenza copper oxide containing respiratory face mask. PLoS One 2010. doi:10.1371/journal.pone.0011295.