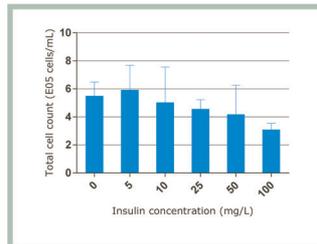
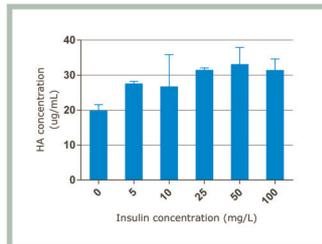


Effect of Insulin on Cell Growth and Virus Production

by Aziza Manceur, PhD



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In an October 2016 webcast, Novo Nordisk Pharmatech hosted a presentation by Aziza Manceur, a research officer at Canada's National Research Council (NRC). Canada's research and technology organization, the NRC celebrated its 100th anniversary in 2016. It serves both the government and private sectors, working with domestic and international clients.

Aziza, who is located in Montreal, presented work performed within the Human Health and Therapeutics Portfolio, which includes about 350 people distributed across four different sites. Their role is to help companies develop and commercialize new health technologies. Members enable companies to de-risk and accelerate the development of products, including vaccines, biologics,

recombinant proteins, and diagnostics. Members also help develop scale-up processes and perform characterization and purification of different products.

Within that portfolio, Aziza's cell culture scale-up team works to produce viral vectors, viruses, antibodies, and recombinant proteins using suspension cell lines in 3- to 500-L bioreactors. Team members are focusing on process robustness, for which they seek to incorporate and apply industrially viable processes.

The presentation that follows was enhanced by the speaker's responses to audience polling results as facilitated by a moderator. A Q&A session concluded the webcast.

The Effect of Insulin on Cell Growth and Virus Production

by Aziza Manceur

The goal of our project was to test insulin as a booster for cell growth and virus production. Insulin was chosen for many reasons. It is already used in cell culture, and it is approved by regulatory agencies. So this is in line with our mission to look for strategies that can be implemented quickly in industry. Insulin also is known for its anti-apoptotic and mitogenic characteristics. We expected that insulin would improve the growth profile of the cells that we work with, but its effect on virus production was unknown.

CELL-LINE GROWTH PROPERTIES

We first examined whether insulin can improve the growth properties of an industrially relevant cell line. We work with a HEK293 cell line that was developed at the NRC. This cell line is grown in suspension in serum-free medium, and a good manufacturing practice (GMP) cell bank is available. Two types of insulin-free media were selected: The in-house medium (IHM), which is a serum-free medium developed at the NRC specifically for this cell line, and the CD293 medium, a chemically defined and a protein-free Gibco medium from Thermo Fisher Scientific.

The gray lines in Figure 1 (TOP ROW) show conditions in the absence of insulin with CD293 medium: The cell density remains below 1.5 million cells/mL with a high cell viability of nearly 100%. But the cell density never reached a density higher than 1.5 million cells/mL. On the

other hand, when we added insulin, we reached a viable cell density of about 6 million cells/mL. We didn't see a significant difference between the two insulin concentrations. So in this scenario, adding insulin alleviated limitations that we had observed initially for the chemically defined medium.

A similar experiment was performed in the IHM medium developed specifically for this cell line (Figure 1, BOTTOM ROW). With or without

Figure 1: Effect of insulin on cell growth in the CD293 medium (TOP ROW) and in IHM-03 medium (BOTTOM ROW); neither shows a significant difference between 10- and 20-mg/L insulin. For the CD293 medium, glucose was added on days 6 and 10, and limitations of the chemically defined medium were alleviated by insulin. For the IHM-03 medium, glucose was added on days 5 and 10, and maximum cell density was reached three days sooner when insulin was added.

● = without insulin ■ = 10 mg/L insulin ▲ = 20 mg/L insulin

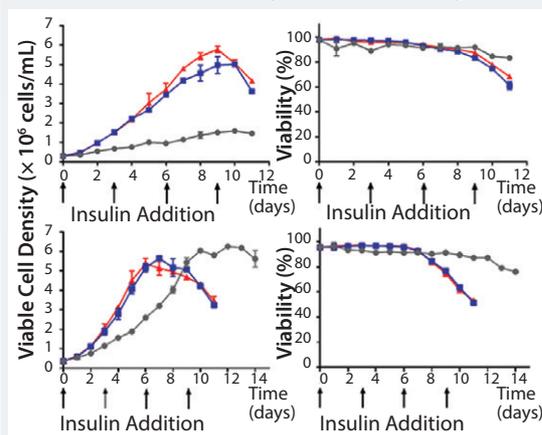
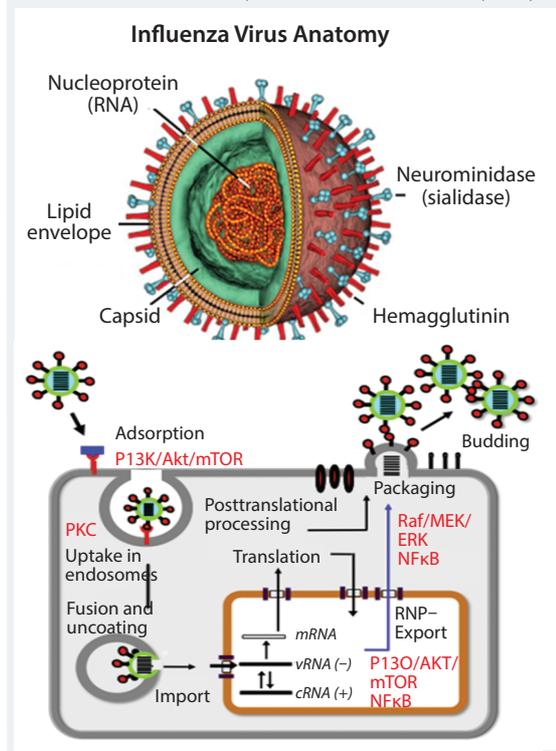


Figure 2: In the influenza virus, insulin is a strong activator of the P13K/Akt pathway and might enhance influenza production (TOP IMAGE from <http://micro.magnet.fsu.edu/cells/viruses/influenzavirus.html>; BOTTOM IMAGE from Planz O., 2013).



insulin we can reach a cell density of nearly 6 million cells/mL. But we can reach that cell density three days sooner when we add insulin (either of the two concentrations tested), thereby accelerating the process. Speeding up the process by three days is a sure benefit in bioprocessing.

Those results showing the effects of insulin on cell growth are positive but not surprising because insulin is a growth factor. Next we decided to examine the potential effects of insulin on influenza production.

ACCELERATING INFLUENZA PRODUCTION

According to the World Health Organization (WHO), the influenza virus kills about half a million people worldwide every year. Currently, vaccinating against the disease is the most effective course of action. About 60% of influenza vaccines are produced by inoculating fertilized chicken eggs, but new production platforms are in development, including using mammalian cell lines. Cell-based vaccine production offers a number of advantages over egg-produced vaccines: It bypasses risks associated with avian flu that could decimate the egg supply; it allows a faster

and more versatile production platform because most strains can be adapted to cells quickly, and it opens the door to vaccine productions free of animal components.

Figure 2 shows what insulin's viral lifecycle looks like in mammalian cells. The multistep process begins with absorption and entry of the virus. Then it releases its viral RNA, which enters the nucleus where more RNA is produced and then exported into the cytoplasm. Finally, new viral particles exit the cells through a budding process. During this replication, the virus takes advantage of several cellular signaling pathways using enzymes and kinases that are part of the cell.

In the diagram, they are shown in red, with the P13K/Akt pathway being implicated in several of those steps. What's interesting is that insulin is a strong activator of the Akt pathway. So when we started this series of experiments, we worked under the assumption that insulin might enhance influenza production by increasing activation of those pathways.

In Figure 3, the *x* axis shows time in hours post infection (HPI). The red line indicates titers measured at different time points. Notice that there is a first viral exit at about eight HPI followed by a second, more important release of viral particles at 16 HPI. That's what we call the *budding process*. Sixteen HPI also is when we start seeing a drop in cell viability, indicated here in black. We therefore measured phosphorylation of Akt in our cells by flow cytometry after infection. The figure shows activation of Akt starting at 15 HPI and ending at 24 HPI, which corresponds to the times of the second viral exit.

EXPERIMENTAL DESIGN

First, several conditions were screened using a 24-well microbioreactor. When cells are infected, trypsin also is added to cleave and activate influenza. Then samples are loaded into the different wells and incubated for 48 hours at 35 °C. At the end of the incubation period, we sample the different wells and determine the viral titer.

We used a multiplicity of infection (MOI) of 0.01, which means that we had one viral particle for every hundred cells. As shown in the template on the left side of Figure 4, four different concentrations of insulin were tested ranging from 5 mg/L to 100 mg/L. Insulin was added at two time points: At the time of infection (TOI) and at

16 HPI, the key time point at which the budding process occurs.

We tested influenza production in the same two media types that we used for cell growth experiments: the in-house medium (IHM) and the CD293 medium. We have also performed the experiments with two different influenza strains, one that belongs to the H1N1 subtypes and a second strain that belongs to the H3N2 subtype.

AUDIENCE POLL 1: METHODS OF CHOICE

The “Audience Poll 1” box shows a wide range of responses, but the hemmagglutination assay earned a majority of the votes for quantifying influenza viruses. The array of responses emphasizes my earlier point that quantifying influenza is not a straightforward task.

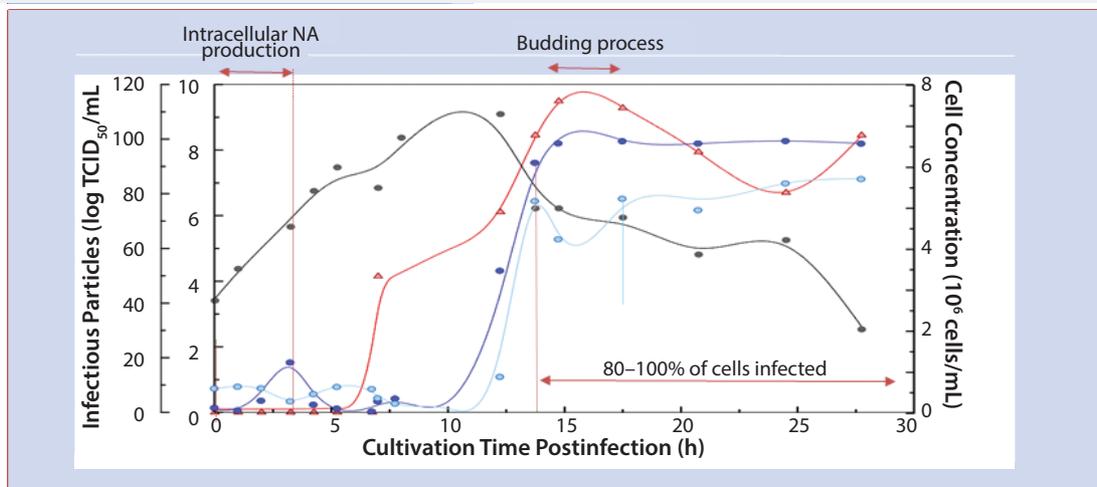
Looking again at Figure 2, you can see two main proteins that are expressed on the surface of an influenza virus: neuraminidase and hemagglutinin (HA). Those proteins are used to identify influenza strains. H3N2 refers to a strain

that expresses hemagglutinin protein from subtype 3 and neuraminidase protein from subtype 2. HA is about four times more abundant than neuraminidase, which is why it is the protein used to quantify influenza. HA comprises two regions: a head region and a stalk region (which is closer to the viral membrane). Most mutations take place in the head region, where most antigenic shift occurs and gives rise to new strains.

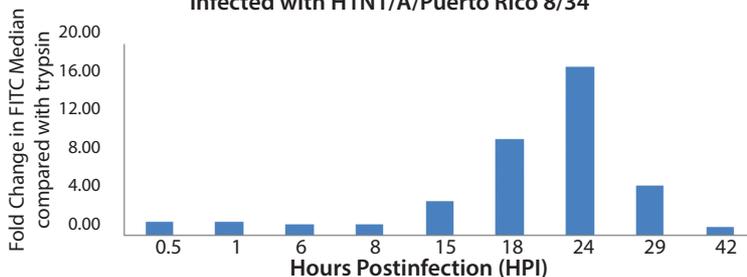
Each strain will require a specific antibody — again, showing the difficulties of quantification.

The good news is that there is a peptide in the stalk region called the *fusion peptide* that is highly conserved across the subtypes and strains because it’s important for virus replication. Sean Li, a collaborator at Health Canada, looked at about 4,000 different strains using bioinformatics tools and determined that this peptide has a very high level of homology among the different strains.

Figure 3: NA protein expression, as described in the text; ● = viable cells, ▲ = infectious particles, ● = intracellular NA, ● = membrane NA



Phospho-Akt modulation in HEK293/SF cells infected with H1N1/A/Puerto Rico 8/34



AUDIENCE POLL 1: METHODS OF CHOICE FOR QUANTIFYING INFLUENZA VIRUSES

Manceur concluded her first set of remarks by saying that anyone who has actually worked with influenza knows that quantifying the virus is actually not at all straightforward. The moderator then presented the audience with a list of techniques from which to choose. Before the polling took place, Manceur commented about those choices.

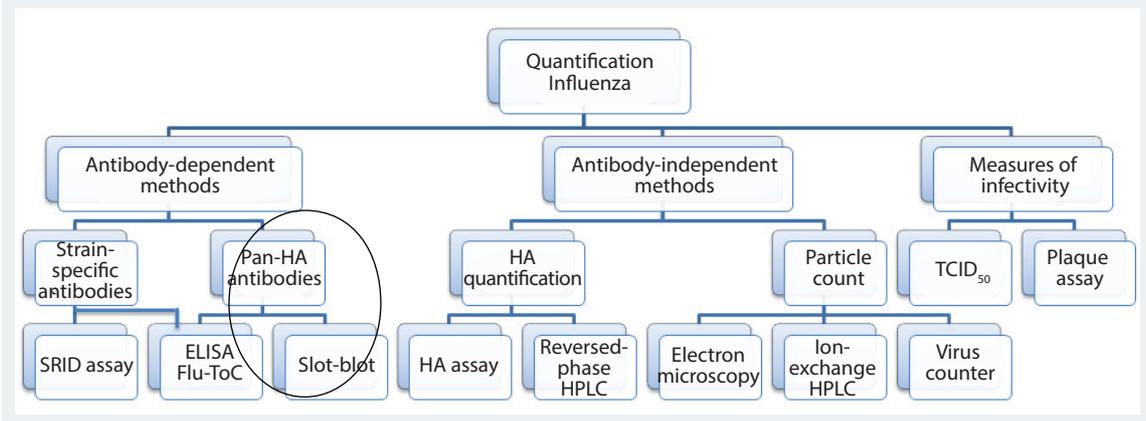
Single Radial Immunodiffusion (SRID), Hemmagglutination (HA), TCID₅₀, and Plaque Assays

Assays: Manceur noted that the SRID assay is the only one that is officially approved by regulatory agencies, so manufacturers always have to run this assay before releasing their vaccines. But because it is low throughput and requires strain-specific antibodies, manufacturers have to use another technique during optimization of vaccine production, such as the HA

assay, which requires red blood cells. TCID₅₀ and the plaque assay mostly measure infectivity.

Electron Microscopy, HPLC or Flow Virometry, ELISA, and Other Immunoblotting Methods: These are mostly physical methods for counting particles. Physical methods such as HPLC and flow virometry are probably the most high-throughput methods that you can perform quickly. The only concern when applying these techniques to mammalian cells is that they also measure nonviral particles such as exosomes, and that can interfere with the readings. Even noninfected cells will give you a count.

Manceur noted that electron microscopy (although expensive) is the gold standard because it is the only method allowing analysts to visually see the viruses for influenza, it expresses proteins at the surface.



Therefore, for quantifying influenza in our laboratory, we have generated pan-HA antibodies that can recognize multiple influenza strains. We have synthesized a peptide-conjugate based on the highly conserved sequence, immunized mice to generate monoclonal antibodies (MAbs), and ended up with two lead candidates. In the Western blot results shown in Figure 5, each lane corresponds to a different HA subtype. A 70-kDa band corresponds to uncleaved HA whereas the band at 25 kDa corresponds to the stem region of HA after cleavage (HA2).

All subtypes are recognized by one or both antibodies. After further analysis, 11H12 was shown to be better at recognizing HAs belonging to Group 1, whereas the second antibody (10A9) seemed to prefer HAs from Group 2. The HA groupings are based on the influenza phylogenetic tree. Combining the two MAbs created a pan-HA cocktail that enabled detection of all the subtypes tested. So far we have tested about 40 different strains of influenza produced

in eggs or cells and even virus-like particles in plants.

For quantification, a Western blot can be used, but it is a low-throughput assay and difficult to optimize. Instead, we chose to use the dot-blot technique. It has a 96-well capacity and is easy to implement with minimal cost. The protocol is quite simple. First we denature the samples with a mild denaturation solution consisting of 4M urea for half an hour to expose the epitope, which tends to be hidden within the virus envelope in the stem region. The samples are then loaded into the wells of the dot-blot apparatus, and vacuum is applied. After blocking for one hour, the membrane is incubated with pan-HA antibodies either overnight at 4 °C or at room temperature for two hours.

Figure 6 shows typical results. The first two columns show calibrating antigens that are loaded in duplicate, with concentrations ranging from 160 ng/mL to 20 µg/mL. Next to that are 10 samples that were also loaded in duplicate, but in



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four different dilutions. Figure 6B shows the standard curve that was generated from the calibrating antigen. If you consider the linear region, the R^2 is 0.98, showing a strong correlation. Then using that standard curve, we quantified the 10 samples in the membrane in 6c, which ranged from almost no HA to 40–50 $\mu\text{g}/\text{mL}$ HA. Many of the samples were not purified, but simply centrifuged.

This technique is reproducible and robust. Some samples were quantified three or four times on different days by different operators and showed a standard deviation below 5%.

Figure 7 shows an H1N1 strain tested in CD293 medium in a microbioreactor. HA concentrations were measured by dot blot, with the blue bars corresponding to results obtained when insulin is added at the time of infection. The grey bars denote when insulin is added 16 HPI. We found a significant increase in HA titer when 25–100 mg/L insulin was added at TOI.

We find it interesting that there is no significant difference in total cell density between the different conditions. This indicates that the increase observed in viral yield is not simply due to an increase in cell density; some other mechanisms are taking place. The maximum increase in HA titer with insulin was about a 1.7-fold increase.

Results with the in-house medium were similar (with the H1N1 strain, Figure 8). The difference was that the increase in yield is observed at lower insulin concentrations ranging from 5 to 25 mg/L both at TOI and 16 HPI, except for one of the

conditions. We saw no difference in the cell counts at time of harvest, which argues against a simple increase in titer resulting from an increase in cell density. Next (Figure 9) we looked at an H3N2 strain in the CD293 medium using the HA assay. As with the H1N1 strain in that cell medium, the increase in yield is observed only when insulin is added at TOI and does not affect the total cell counts at the time of harvest.

If you pool all results from the two different media with the two different influenza strains, it

Figure 4: Experimental plan for influenza production

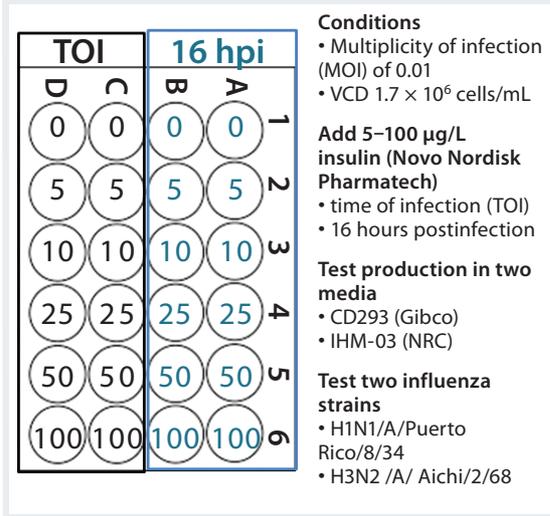
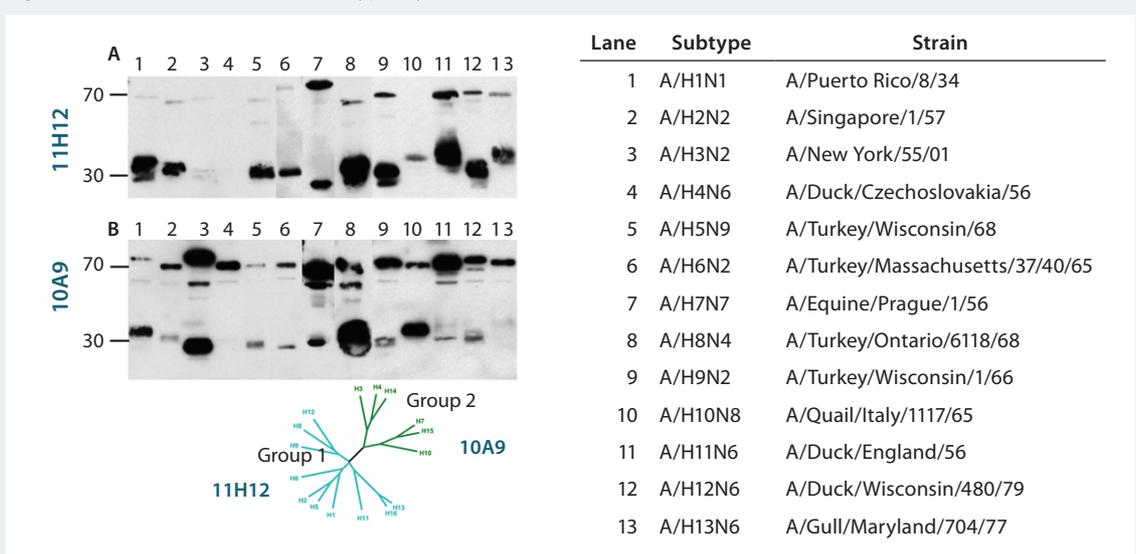


Figure 5: Detection of influenza A subtypes by Western blot



seems that the best condition is to add 25 mg/L insulin at TOI. That appears to increase the viral yield in all the different conditions used.

AUDIENCE POLL 2: EXAMINING THE MECHANISM OF ACTION

Based on the results of the second audience poll (see the “Audience Poll 2” box), I should say a few things about the mechanism of action (MOA). My guess is that insulin should work with other cell lines, especially with similar viruses. Influenza is an enveloped virus that buds out of a cell. So other viruses that work through similar mechanisms — such as lenti- and

retroviruses, which are also enveloped — could probably benefit from using insulin. Other viruses such as adenoviruses use a different mechanism to exit infected cells. Insulin might still assist in their production, but it would be through a different MOA than for influenza.

INCREASING INFLUENZA PRODUCTION

To continue my thoughts about how insulin mediates an increase in influenza production, we looked at the phosphorylation of Akt and mTOR using flow cytometry. In this experiment, insulin was added at 16 HPI. The control consisted of cells treated with trypsin only, but not infected with influenza. We calculated the ratio of phospho-Akt and phospho-mTOR from infected cells over the one from noninfected cells. Both kinases were activated by H1N1 starting at 15 HPI. That activation is stronger in the presence of insulin,

AUDIENCE POLL 2: INSULIN’S EFFECT ON OTHER CELL LINES

The moderator asked, “Does insulin affect the viral production in other cell lines or viral expression systems?” and listed the following choices for audience consideration:

- Cell lines (e.g. Vero, Per.C6, MDCK, EB66)
- Other virus expression vectors (retrovirus, lentivirus, adenovirus)
- Both of the above
- None of the above

The audience voted for “Both of the above.” This led to discussion of mechanism of action because improvements in yield could be specific to the cell line or virus strain.

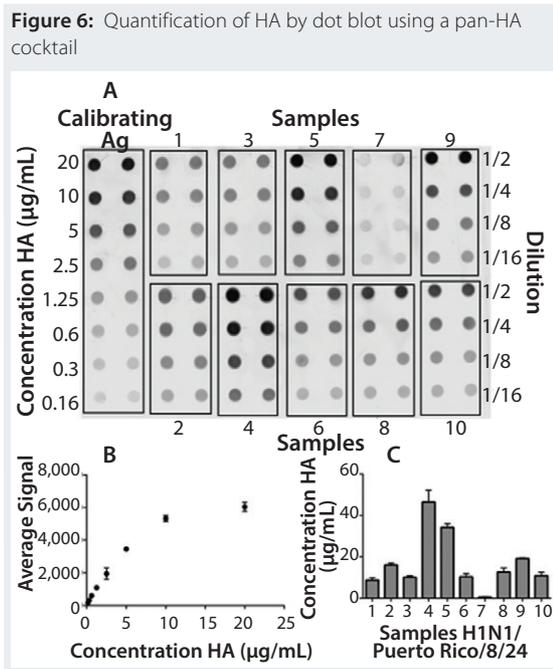
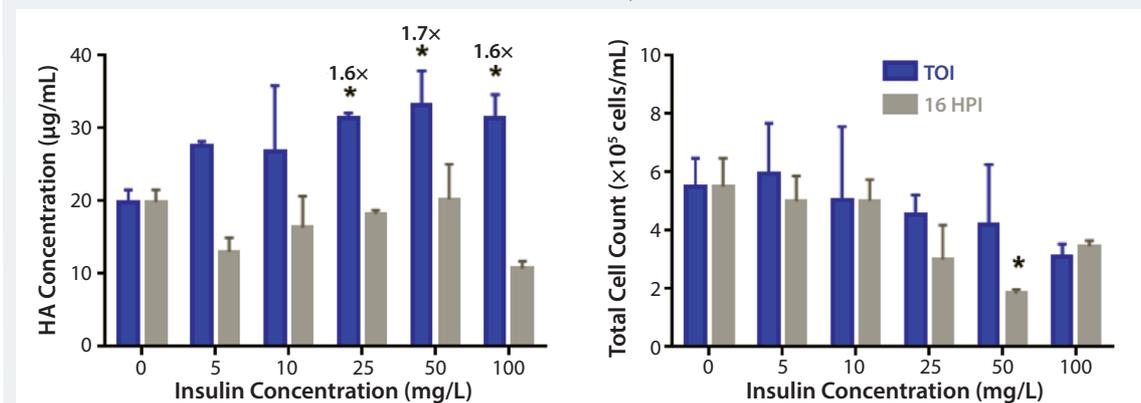


Figure 7: H1N1 A/Puerto Rico 8/34 production in CD293 media (in microbio reactor format); total HA production 1.6-fold increase with 25 µg/mL insulin at TOI, 1.7-fold increase with 50 µg/mL insulin at TOI, and 1.6-fold increase with 100 µg/mL insulin added at TOI. Increase in HA content was not attributable to increased cell density.





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which makes sense because it is an activator of this pathway.

The two kinases play different cellular roles. mTOR is involved in protein synthesis. So increased mTOR activity indicates an increased protein synthesis. We do see an increase starting 15 HPI and up to 24 HPI, but that increase is the same with or without insulin. The difference is not statistically significant. So activation of mTOR is probably not the mechanism behind the effect of insulin on viral production.

Akt, on the other hand, is strongly activated by insulin infection with a 10- to 20-fold increase in phosphorylation. But additional insulin further increases that activation, especially at 18 HPI. Akt activation is associated with several cellular activities. Akt has nearly 100 cell substrates, and it also is tightly associated with increased cell survival and

reduced apoptosis. So this could be the mechanism by which insulin enhances viral yield.

We did measure cell viability, but only at the time of harvest (48 HPI). The viability was not increased by insulin, but insulin possibly could increase the cell survival at critical points between 18 and 24 HPI, during the budding process. That would allow production of more viral vesicles and delay apoptosis. So that is one possibility: that insulin, by increasing Akt activity, also would delay apoptosis and therefore allow the release of more viral particles during the budding process.

That theory is further supported by some literature showing that a protein produced by influenza (NS-1, a nonstructural protein) played

Figure 8: H1N1 A/Puerto Rico 8/34 production in IHM-03 (in microbioreactor format); total HA production 1.7-fold increase with 5 µg/mL insulin at TOI or 16 hours postinfection (HPI), 1.6-fold increase with 10–25 µg/mL insulin at 16 HPI, and 1.9-fold increase with 25 µg/mL insulin added at TOI. Increase in HA content was not attributable to increased cell density.

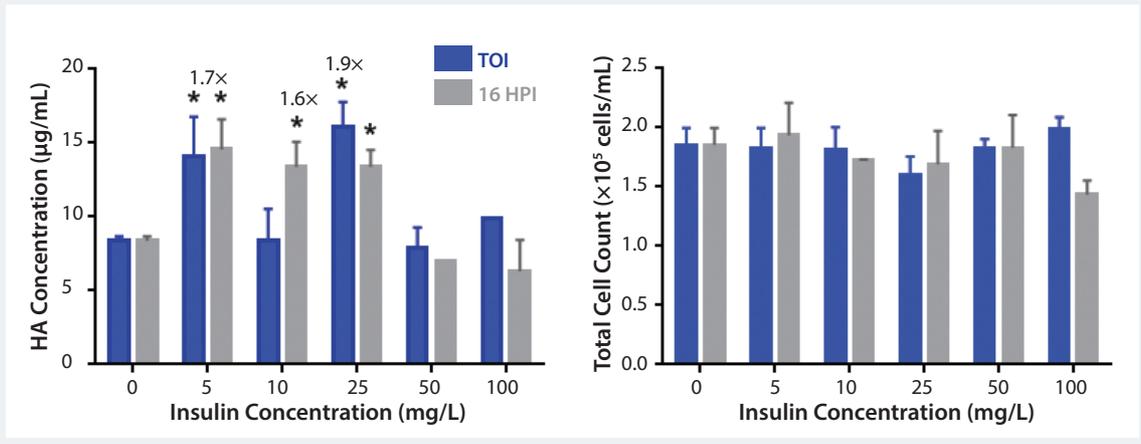
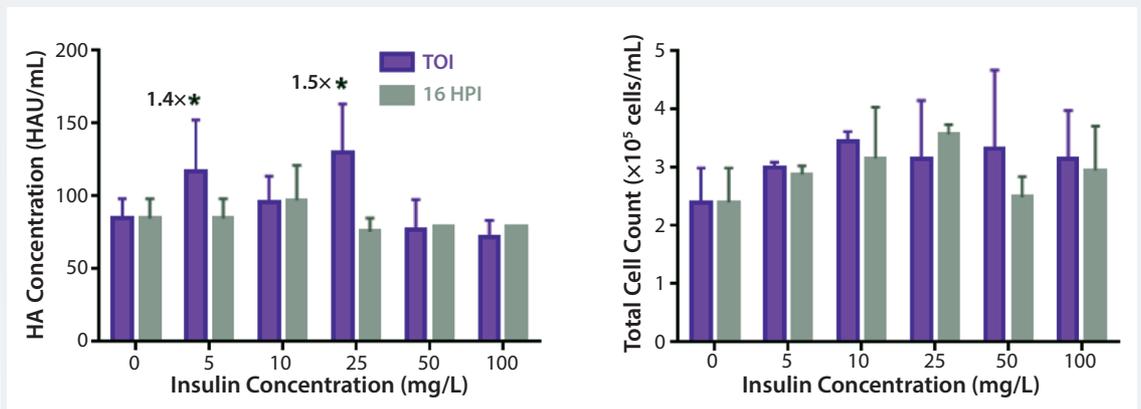


Figure 9: H3N2 A/Aichi/2/68 production in CD293 media and quantified by HA assay; total HA production for H3N2 strain was 1.4-fold increase with 5 µg/mL insulin at TOI and 1.5-fold increase with 25 µg/mL insulin at TOI. Increase in HA content was not attributable to increased cell density.



BIOREACTOR PARAMETERS: SCALABILITY

Goal: Compare production of influenza in a 7-L bioreactor (Applikon) and in 50-mL shake flasks

Internal control: Infected cells from the bioreactor right after infection

External control: Cells were infected in a 50-mL shake flask and were never in the bioreactor

Parameters:

100 rpm, pH 7.15, 40% O₂

Temperature: 35 °C after infection

Inoculation density: 0.25 × 10⁶ (Friday)

Density: 2.3 × 10⁶ cells/mL (at time of infection, three days postinoculation)

Cell viability: 97% at time of infection

Density: 2.99 × 10⁶ cells/mL at time of harvest

Cell viability: 80% at time of harvest

Harvest time: 48.5 hpi

exactly that role in the cells. NS-1 leads to activation of Akt and a decrease in apoptosis. If you follow the theory, it means that insulin basically mimics the role of NS-1.

Also interesting is that NS-1 is strain specific. All influenza strains have different types of NS-1. We compared Akt activation in cells infected by the two different strains and found that Akt is more highly activated by the H1N1 strain than the H3N2 strain. So it seems that H1N1 expresses an NS-1 protein that leads to higher phospho-Akt: higher Akt activation, higher production, and less apoptosis.

SCALING UP PRODUCTION

The cell scale-up team wanted also to see whether what was observed in small-scale and microbioreactor format could be reproduced at larger scale. Team members compared production of influenza in a 7-L bioreactor with productions in 50-mL shake flasks.

Two controls were used for this experiment. An internal control consisted of cells obtained from the bioreactor after infection (a 50-mL sample). An external control was of the same cells infected in parallel in a shake flask, so those cells had not been in contact with the bioreactor.

The “Bioreactor Parameters box” shows conditions used to run the bioreactor (the same as in the shake flask.): 35 °C with an MOI of 0.01 and a harvest time at 48.5 HPI. The results showed about 13 µg/mL HA in the reactor compared with 14.9 and 14.8 in the shake flasks. So the team could scale-up the process and obtain

the same results in the bioreactor as in the shake flask. The effect of insulin on influenza production in a large-scale bioreactor is currently being evaluated.

The team also is interested in how insulin could affect production of other viruses, such as lentiviral vectors, retroviruses, adenoviruses and adeno-associated viruses (AAV). They are hopeful that the similar treatment and condition would also help increase the production of lentiviral vectors and retroviruses especially because they go through a similar budding process as influenza. For adenoviruses and AAV, the mechanism is likely to be different because the viruses go through a lytic process in which they basically tear open cells to be released. This work is ongoing.

INCREASING YIELD, ACCELERATING CELL GROWTH

During cell growth we were able to increase maximal cell density by adding insulin to the chemically defined medium. And even in medium that was well-suited for cells developed in-house, we could accelerate cell growth by adding insulin.

Although we have worked mostly with influenza so far, we have found that regardless of the strain or medium type, adding insulin at the time of infection increases yield by about two-fold for the H1N1 and about 1.5-fold with the H3N2 strain. The implication for manufacturing is that if you double the amount of HA produced, you can also reduce the number of runs by two. That is a cost-effective way of manufacturing.

AUDIENCE QUESTIONS AND ANSWERS

Why was insulin added every 72 hours during the cell growth experiments? We picked that time

because the half-life of insulin is about 72 hours. We didn't test too many other scenarios, so it is quite possible that fewer additions or even lower concentrations of insulin also could be tested, and it might depend on the cell type.

Did insulin significantly change glucose consumption in the cultures? Yes. When we started, the glucose was around 20 mM. Whenever it dropped lower than 8 mM, we added glucose. Without insulin, we added glucose only once, on day 10. But in the presence of insulin, we had to add insulin twice, at day five or six and day 10.

Which parameters were controlled in the microbioreactor? We were able to control oxygen levels at 40% throughout the whole experiment.

The implication for manufacturing is that if you double the amount of HA produced, you also can **REDUCE the number of runs by two. That is a cost-effective way of manufacturing.**

The temperature also was maintained at 35 °C and the pH at 7.2, but only through the addition of CO₂. With the apparatus we had, we were unable to add a base, such as NaOH.

How does the dot-blot method compare with other quantification methods? When we perform the dot blot, we are measuring the total HA concentration. So the best technique to compare it with is the HA assay, the one with the red blood cells, because that also measures total HA concentration.

We analyzed about 50 different samples and looked at the correlation between the two techniques. There is good correlation between the dot blot and the HA assay with an R² of about 0.8. The greatest differences observed were at high HA concentrations. The advantage of using the dot-blot assay instead of the HA assay is that you don't have to rely on the availability of red blood cells from chickens. With the dot-blot assay you can run your assay whenever you want to.

I've also found that the dot-blot assay is more reproducible than the HA assay because in the latter you need to have a specific concentration of red blood cells to get hemagglutination.

However, when I tried to make similar comparisons with the single radial immunodiffusion (SRID) assay, the correlation was not that strong. In that assay, you actually measure the trimers that form the precipitation ring. So the correlation is not that good because we are not measuring the same thing. In the SRID assay we measure the trimeric form of HA, and with the dot blot we measure the total amount of HA.

Why would one antibody bind preferentially to Group 1 HA and the other one target Group 2?

Although we have not looked specifically at this issue, the homology for 12 amino acids out of the 14-amino-acid peptide used to generate the antibodies is about 99.9%. For the two remaining amino acids, the homology level is only 60–70%.

Usually an antibody will bind to a peptide of 8–10 amino acids. It is quite possible that one antibody was skewed toward one end of the peptide and the other skewed toward the other end, or that the difference is due to the two amino acids with less sequence homology. That might explain the different binding affinity of the antibodies to different HA subtypes. But actual binding studies are in progress, after which we'll have a clearer answer.

Glycosylation also is different between the subtypes. That might explain why one antibody is better for one group than the other.

Is there a restriction in using insulin for a parenteral bioproduct? You need to check that with regulatory agencies. But insulin is animal-free and well characterized, and it can be produced in yeast or bacteria. As long as you document it and you know the source of your insulin, you should be fine.

Did you look at viral titers in addition to the HA content? We ran TCID₅₀ assays for that best condition of 25-mg/L insulin. The trend is that it does seem to increase the TCID₅₀ titer, but that assay has so much variation that we ended up with high standard deviations. The TCID₅₀ titer was increased when we added insulin, but that increase was not significant when we ran statistical assays because of the standard deviation between the different replicates. We need to rerun both assays to be sure, but we did see an increase in the HA content. 🌐

ACKNOWLEDGMENTS

The speaker thanks her colleagues at NRC — Sven Ansoerge, Sonia Tremblay, and Rhonda Kuo Lee — as well as Clare Medlow and Magnus Franzmann from Novo Nordisk Pharmatech for their scientific expertise and for providing the insulin. *These results described do not represent an endorsement of any product by NRC.*

Aziza Manceur, PhD, is a research associate at National Research Council Canada (aziza.manceur@nrc.ca, 6100 Royalmount, Montreal, Quebec, H4P 2R2, Canada). To access this webinar directly, please visit www.business-review-webinars.com.

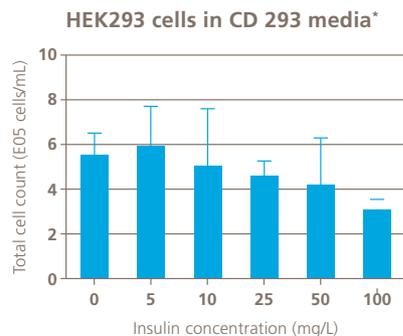
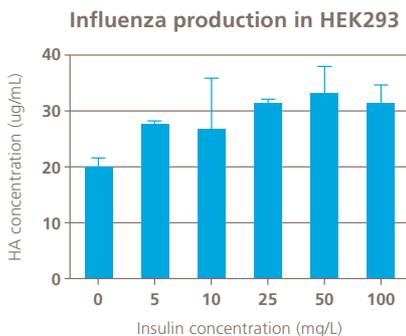
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*Data kindly supplied by Aziza Manceur, National Research Council Canada. Hemagglutinin (HA) assay is used for quantification of Influenza (H1N1). Insulin Human AF used in the experiments is supplied by Novo Nordisk Pharmatech. CD 293 media is trademark of Thermo Fisher Scientific.