Molecular Therapy
Methods & Clinical Development
Original Article

NaCl and KCl mediate log increase in AAV vector particles and infectious titers in a specific/timely manner with the HSV platform

Chenghui Yu,[1,2,3] Prasad D. Trivedi,[1,3] Payel Chaudhuri,[1] Radhika Bhake,[1] Evan J. Johnson,[1] Tina Caton,[1] Mark Potter,[1] Barry J. Byrne,[1] and Nathalie Clément[1]

[1]Department of Pediatrics, Powell Gene Therapy Center, University of Florida, 1200 Newell Drive, Academic Research Building, RG-187, Gainesville, FL 32610, USA; [2]State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200438, China

The increasing demand for adeno-associated virus (AAV) vectors, a result from the surging interest for their potential to cure human genetic diseases by gene transfer, tumbled on low-performing production systems. Innovative improvements to increase both yield and quality of the vector produced have become a priority undertaking in the field. In a previous study, we showed that adding a specific concentration of sodium chloride (NaCl) to the production medium resulted in a dramatic increase of AAV vector particle and infectious titers when using the herpes simplex virus (HSV) production system, both in adherent or suspension platforms. In this work, we studied additional salts and their impact on AAV vector production. We found that potassium chloride (KCl), or a combination of KCl and NaCl, resulted in the highest increase in AAV vector production. We determined that the salt-mediated effect was the most impactful when the salt was present between 8 and approximately 16 h post-infection, with the highest rate increase occurring within the first 24 h of the production cycle. We showed that the AAV vector yield increase did not result from an increase in cell growth, size, or viability. Furthermore, we demonstrated that the impact on AAV vector production was specifically mediated by NaCl and KCl independently of their impact on the osmolality of the production media. Our findings convincingly showed that NaCl and KCl were uniquely efficacious to promote up to a 10-fold increase in the production of highly infectious AAV vectors when produced in the presence of HSV. We think that this study will provide unique and important new insights in AAV biology toward the establishment of more successful production protocols.

INTRODUCTION

The past decade has witnessed a surging interest for recombinant adeno-associated virus (rAAV) vectors for their high potential to cure human genetic diseases by gene therapy. There are currently more than 125 clinical trials listed on the ClinicalTrials.gov website, and the success of AAV vectors in the clinic led to three commercial products in recent years, Glybera (uniQure), Luxturna (Spark Therapeutics), and Zolgensma (Avexis).[1] Several additional drug products are poised to be marketed in the next 5 years. The exponentially increasing demand for these vectors has triggered one of the most dynamic and productive research times for AAV vectors, with a strong emphasis on the establishment of robust and scalable protocols for the production of high titer stocks, both in academic settings, where it originated, and in industrial settings. However, the challenge is yet to be fully tackled, and generating the doses of vectors needed to conduct pre-clinical and clinical protocols still hampers AAV vector clinical development.[2,3] Since the interest in AAV vectors is relatively young in industrial settings, traditional manufacturing parameters established for other biologics such as monoclonal antibodies (mAbs)[4] or recombinant proteins have yet to be fully studied in large-scale formats, and previously identified factors for cell growth or protein production may not necessarily apply to the production of a more complex biologic such as the AAV vector, composed of a DNA genome and a protein coat, and whose life cycle is tightly regulated by cellular and helper viral functions. For these reasons, further improvements to increase the yield as well as the quality of the vector produced remain a priority undertaking.

AAV is a parvovirus of the subgroup dependo-virus, in that it relies on a helper virus to replicate and produce progeny in permissive cells. Adenovirus (Ad) has historically been the most commonly utilized helper virus to produce the wild-type virus (WT-AAV), with the vast majority of the current standard protocols incorporating the five well-defined Ad5 gene products required for AAV replication, namely E1A/B, provided by the producer cells human embryonic kidney cells, HEK293, E2A, E4ORF6, and virus-associated (VA) RNAs[5–7] (and reviewed in Clément and Grieger[2]). Although herpes simplex virus (HSV) was less popular in the early days of AAV production, the relationship between WT-AAV and HSV type 1 (HSV-1) has been

Received 11 September 2020; accepted 15 February 2021;
https://doi.org/10.1016/j.omtm.2021.02.015.

[3]These authors contributed equally

Correspondence: Nathalie Clément, Department of Pediatrics, Powell Gene Therapy Center, University of Florida, 1200 Newell Drive, Academic Research Building, RG-187, Gainesville, FL 32610, USA.
E-mail: nclement@peds.ufl.edu

well studied, and HSV-based production platforms in mammalian cells have gained further attention in recent years.[8,9] Six HSV-1 gene products have been identified as critical to support AAV replication, namely UL5, UL8, UL52, ICP8 (UL29), UL30, and UL42, and three additional ones, ICP0, ICP4, and ICP22, can further enhance AAV replication[10] (and reviewed in Clément et al.[8]). Built within a small single-stranded DNA genome of approximately 4.7 kb (reviewed in Salganik et al.[3]), WT-AAV-2 encodes for a total of nine major proteins, four Rep proteins (Rep78, 68, 52 and 40), which are essential for the genome rescue, replication, integration, and regulation of AAV gene expression, and three main capsid proteins (Cap) (VP1, VP2, VP3) that form the viral capsid and differ for each AAV serotype, as well as two recently identified capsid proteins, the assembly-activating protein (AAP) and the membrane-associated accessory protein (MAAP). Typically, AAV production is triggered by providing these nine proteins in *trans*, using a plasmid DNA (transfection methods) or a helper virus genome such HSV or the baculovirus (BV) (infection methods).[11] The rAAV genome only retains the inverted terminal repeats (ITRs) from WT-AAV-2, which act as a viral origin of replication and are required for rescue, replication, and packaging of the recombinant genome.

In our previous work, we described our AAV production platform using HSV-1 to generate high yields of AAV vector stocks.[9,12] In this system, production is initiated by co-infecting HEK293 cells with a recombinant HSV (rHSV) carrying the AAV genome of interest (HSV-GOI) and the HSV carrying the AAV Rep and Cap functions (HSV-RC) at an established multiplicity of infection (MOI). In a previous study, we showed that adding a specific concentration of sodium chloride (NaCl) to the production medium could increase AAV titer by more than 1 log both in an adherent or suspension HEK293 cells format, resulting in specific yield superior to 1×10^5 vector genomes (vg)/cell. Critically, the rate increase observed in AAV vector genomes was fully correlated with an increase in the infectious titers or the vectors transducing capability. In the current work, we first aimed to extend the scope of salts that could be used as culture additives to increase AAV vector production. Second, we wanted to assess whether the salt-mediated osmolality change in the production medium was a possible cause to trigger such an effect, as reported for the production of mAbs.[4] We found that potassium chloride (KCl) was alone or in combination with NaCl sufficient to reproduce the effect previously observed with NaCl and that both salts operate in a similar timely fashion during the production cycle. We did find a correlation between salt-mediated osmolality values as well as glucose-mediated osmolality, but the impact of osmolality alone was not sufficient to fully reproduce the rate of increase observed with NaCl or KCl. Our data convincingly show that NaCl and KCl have unique properties in triggering a dramatic yield increase in AAV vector production when using the HSV system.

RESULTS

KCl significantly increases volumetric and specific AAV yields

Based on data generated during our original study that revealed a dramatic impact of NaCl on AAV vector yield, we continued our evaluation by testing a series of salts, mostly from the chloride family. During our initial screenings, we found that KCl had the most significant effect on AAV vector production. Data for the other salts are presented later in this section. In the first experiment, a quick screening was performed between 20 and 100 mM KCl added directly to the production medium and concurrently to the addition of rHSV-GFP and rHSV-AAV2/9. Vector genome titers and infectious titers (or transducing unit [TU] titers) were assessed in the Benzonase-treated crude lysates generated from the various conditions. We found that KCl triggered a dose response with an increase in AAV vector particles produced between 20 and 60 mM when compared to the production in non-treated cells (Figure 1A). Concentrations above 80 mM resulted in a dramatic decrease in AAV vector production and were excluded from further evaluation. We established a more thorough concentration range and tested KCl concentrations between 10 and 60 mM, as illustrated in Figure 1B. We observed that the most significant effect on AAV vector production was obtained with approximately 40 mM KCl supplemented, with an optimal concentration range between 30 and 50 mM. Notably, a modest effect was detected at a concentration as low as 10 mM. Concentrations above 50 mM resulted in a decline of the rate increase. Within each individual experiment, we calculated the fold change, or ratio, between the total vector genomes obtained for each condition with the total amount obtained in the non-treated cells (set as value of 1) and report the averaged values in Figure 1C. The average increase in vector genome titer was approximately 10-fold when compared to the non-treated condition (Figure 1C, 40 mM). The overall KCl-mediated effect was also confirmed at the level of infectious particles in these experiments. Importantly, the increase in particle titers was strongly correlated with an increase in transducing unit titers, with an average of a 20-fold increase in transducing unit titers at 40 mM over the control conditions (Figures 1B and 1C).

Next, we compared the effect of KCl and NaCl in side-by-side experiments, with KCl supplemented at 40 mM and NaCl at 60 mM, as previously established.[12] It is noteworthy that because the HSV stocks were formulated in a production medium that contained 600 mM NaCl, the final concentration of NaCl to be added to the production medium needed to be adjusted accordingly. As shown in Figure 1D, the average vector genome yield was $1.04 \times 10^{13} \pm 6.20 \times 10^{12}$ vg/lysate with KCl supplementation, $1.01 \times 10^{13} \pm 6.99 \times 10^{12}$ vg with NaCl supplementation, as compared to $1.27 \times 10^{12} \pm 6.52 \times 10^{11}$ vg without salt supplementation. Despite the variability in titers across the numerous experiments, the increase in both vector genome and transducing unit titers was found to be statistically significant. In Figure 1E, the results from the same experiments are represented by the value of the increase in titers for each condition versus the non-supplemented condition (Figure 1E). The average particle yield increase was 8.50 ± 2.56-fold for KCl and 7.19 ± 2.82-fold for NaCl. This increase was similar to the one previously observed with NaCl.[12] As noted earlier, the vector genome yield was highly correlated with the transducing unit yield, with an average increase in transducing units of 11.97- and 7.58-fold for KCl and NaCl, respectively (Figures 1D and 1E). This resulted in reduced vector

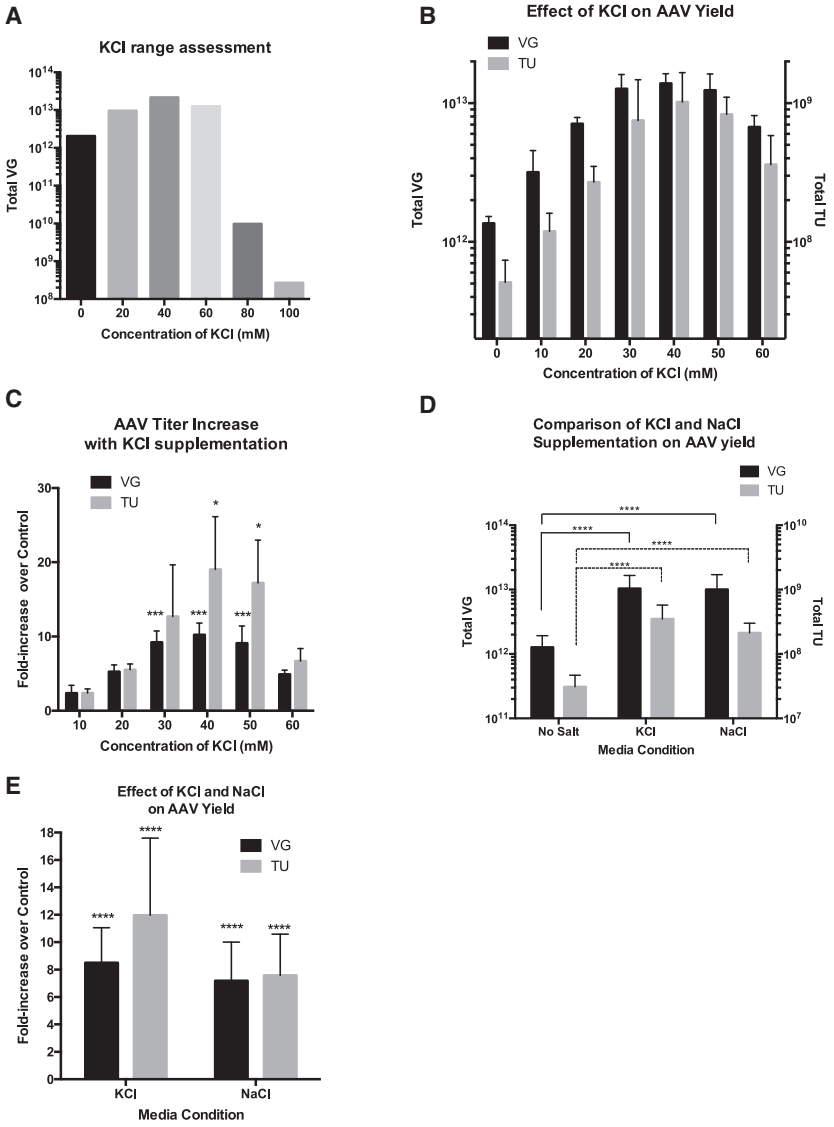

Figure 1. Effect of potassium chloride (KCl) on AAV vector titers and comparison to sodium chloride (NaCl)

(A) In this single experiment (n = 1), a quick screen of various KCl concentrations was performed by adding KCl at the time of cell infection from 20 to 100 mM final and compared to the non-treated cells (Control). Total vector genomes (vg) were obtained by quantitative real-time PCR from cell lysates for each condition performed once. (B) Cells were infected with rHSV-GFP and rHSV-AAV2/9 in the presence of various concentrations of KCl (50 mL production volume, 5×10^7 cells). Total vector genome particles (black bars) and transducing units (TU, gray bars) were assessed in cell crude lysates. The experimental run was repeated three independent times and values represent the average of the three independent experiments and their standard deviation. (C) The total vector genomes and infectious units from the experiment described in (B) are expressed as the fold increase ratio versus the non-treated cells (controls). The values show the average and standard deviation of the ratios calculated from three independent experiments (n = 3, same as shown in A). Statistical differences were evaluated for each condition versus the controls. *$p < 0.05$, ***$p < 0.001$. (D) Cells were infected in the presence of KCl or NaCl at their optimal concentration (40 and 60 mM, respectively) concurrently to untreated cells (no slat). The figure shows the averages of total vector genome values and total transducing unit values obtained from n independent experiments: controls and KCl, n = 37; NaCl, n = 19. Statistical differences were calculated for each group versus their paired controls. ****$p < 0.0001$. (E) Same experiments as in (D) with the total vector genomes and total transducing units expressed as a fold increase of KCl- or NaCl-treated conditions versus their paired control (non-treated) in each independent experiment, and averaged. Statistical differences were calculated for each group versus their control (set as a value of 1 in each experiment). ****$p < 0.0001$.

genome-to-transducing unit ratios, a measure often used to express AAV vector potency, with lower values reflecting an increase in potency when assessed *in vitro*.

To verify that the vector genome titer increase observed was not due to the presence of possible traces of KCl in the cell crude lysates tested, which could affect the PCR reactions, we prepared a mock cell lysate without HSV/AAV but with KCl supplementation and harvested similarly to the AAV-containing samples. We used this virus-free cell lysate to spike-in a known amount of a purified AAV stock submitted to quantitative real-time PCR quantification. We noted that the cell lysate did not impact the overall result of the quantitative real-time PCR and that the vector genome increase was consistent with an increase in AAV vector genome titer (data not shown). Similar to the quantitative real-time PCR assay, we verified that possible traces of KCl present in the cell lysate did not affect the transduction efficiency of the rAAV in our *in vitro* assay (data not shown). Lastly, we prepared two 3-L scales of fully purified stocks of rAAV9-GFP in the presence of either KCl or NaCl supplementation. Both conditions yielded nearly identical titers for both vector genomes and transducing units at each step of the downstream purification procedure (see Table 1). Importantly, the vector genome-to-transducing unit ratio was 1.10×10^4, similar to the ratios obtained in small-scale experiments. This further confirmed that the increase in potency observed in crude lysates was not due to potential impurities in the virus preparations, and that KCl and NaCl triggered a very similar AAV vector production yield increase.

Combinations of NaCl and KCl or phosphate-buffered saline (PBS) also increase AAV vector titer

We next evaluated whether the combination of NaCl and KCl could act in synergy to further increase the overall AAV vector yield. The concentration of NaCl supplemented varied between 16 mM (only salt from the HSV stocks, or 26.7% of optimal target) and 60 mM

Table 1. Comparison of NaCl and KCl supplementation at large-scale production

	KCl supplementation	NaCl supplementation
Total vg harvest	2.77×10^{15}	2.54×10^{15}
Total vg of purified dialyzed stock	5.83×10^{13}	4.78×10^{13}
vg/cell harvest	9.23×10^{5}	8.47×10^{5}
vg-to-TU ratio	1.10×10^{4}	1.10×10^{4}

AAV9-GFP was produced side by side and purified from a 3-L scale production in the presence of NaCl or KCl supplementation; n = 1 for each salt. vg, vector genome; TU, transducing unit.

(100% of target), and the concentration of KCl varied in opposite order from 0 mM (0% of target) to 40 mM (100% of target). One condition had both salts added at 100% of target concentration (60 mM NaCl and 40 mM KCl). As shown in Figure 2A, no significant improvements were observed with any of these conditions when compared to each salt used individually, and the condition with both salts added at their optimal concentration resulted in a significant decrease in AAV vector yield. Although the differences were not significant, we did note an upward trend when KCl concentration was increased. Collectively, these data suggested that each salt could produce the maximal impact individually and without a benefit when combined. Based on these data, one could hypothesize that NaCl and KCl act on the same cellular targets, which could also explain a "saturation" effect.

Next, we evaluated Dulbecco's PBS (DPBS), a commonly used buffer comprising a cocktail of various salts at various concentrations, including NaCl and KCl. Using a 10-fold concentrated DPBS, we tested final concentrations ranging from 0.1× to 0.5× added into the production medium and assessed the AAV vector yield as previously described. We observed a significant increase at concentrations of DPBS of 0.2× and 0.3× (Figure 2B), but the effect of KCl or NaCl alone, performed side-by-side, was consistently more pronounced. At 0.2× and 0.3× DPBS, the final concentration of NaCl and KCl supplemented would be approximately 27 and 41 mM for NaCl, and 0.54 and 0.78 mM for KCl. This could explain why DPBS could only partially reproduce the effect mediated by NaCl, while KCl was in an amount well below the optimal concentration required for AAV vector production increase.

Other chloride or phosphate salts have little to no effect on AAV vector production yield

In an attempt to further explore the potential role of monovalent cations (Na^+, K^+) or anions (Cl^-), we evaluated as series of other phosphates and chloride salts. We noted a dose curve effect when using potassium phosphate dibasic (K_2HPO_4) and sodium phosphate dibasic (Na_2HPO_4) between 1 and 4 mM, which was sustained up to 10 mM (Figure S1). Concentrations above 10 mM for K_2HPO_4 and 20 mM for Na_2HPO_4 were toxic to the cells and resulted in a drastic reduction in AAV vector production. Overall, however, the effect was relatively modest with an approximately 2.5-fold AAV vector particles increase, and consistently lower than the increase obtained for NaCl and KCl in these paired experiments (Figure S1). The effect of potassium phosphate monobasic (KH_2PO_4) was notable but not significant (Figure S1). These data suggested that providing K^+ and Na^+ from another source than NaCl and KCl could not fully reproduce the increase triggered by these two salts, suggesting that Cl^- could contribute to the effect.

Next, we evaluated a series of other chloride salts, and the data are summarized in Figures S2A–S2G. The presented data resulted from a quick and wide range of screening and should not constitute any definitive conclusion on any of the salts with regard to AAV vector production, but rather they present a body of data to build on future evaluation. Among the salts tested, zinc chloride ($ZnCl_2$), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), cobalt chloride, and manganese chloride ($MnCl_2$) showed a modest but not significant increase (below a 1.5- to 2-fold increase) within a defined concentration range. For most of them, however, we also reached a concentration range above which the AAV vector production was reduced, whether from cell toxicity or other effects not investigated in this study. At some concentrations, $CaCl_2$ and $ZnCl_2$ triggered the formation of a precipitate in the culture medium that was likely responsible for the loss in AAV. The last two other salts were evaluated, that is, sodium selenite (Na_2SeO_3) and iron nitrate, and neither had a beneficial effect on AAV vector production before reaching a concentration that led to a dramatic reduction in the AAV vector titer. Our data collection to date showed that the sole use of KCl, or NaCl, is sufficient to mediate up to a 10-fold increase in AAV vector production yield and could not be fully reproduced by any other salts tested, notably among other potassium or sodium salts and chloride salts.

Increase in AAV vector titers is not correlated with cell growth or improved HSV infection

We wanted to verify whether the increase in AAV vector titers triggered by salt supplementation could be due to an increase in either the overall cell concentration or viability, or whether there was an effect on the overall cell size, which could reflect a change in the cell metabolic state. We checked the cell concentration, viability, and size at various time points during the course of an AAV vector production with rHSV. We observed that the total cell number was reduced in KCl- and NaCl-treated conditions (Figure 3A), which was also be observed by the naked eye with consistently smaller cell pellets upon harvest. Similarly, the cell viability as well as the cell size were decreased in the salt-treated conditions (Figures 3B and 3C), likely due, at least in part, to viral cytotoxicity. These findings supported our conclusion that the increase in AAV vector yield was the direct result of an increase in AAV vector particles produced per cell rather than an increase in cell number.

KCl supplementation did not significantly affect the infection rate of rHSV, as we had previously documented for NaCl. To confirm this hypothesis, we infected cells with rHSV-GFP in the presence or absence of KCl and NaCl and determined the percentage of GFP-expressing cells shortly after exposure. Figure 3D documents that

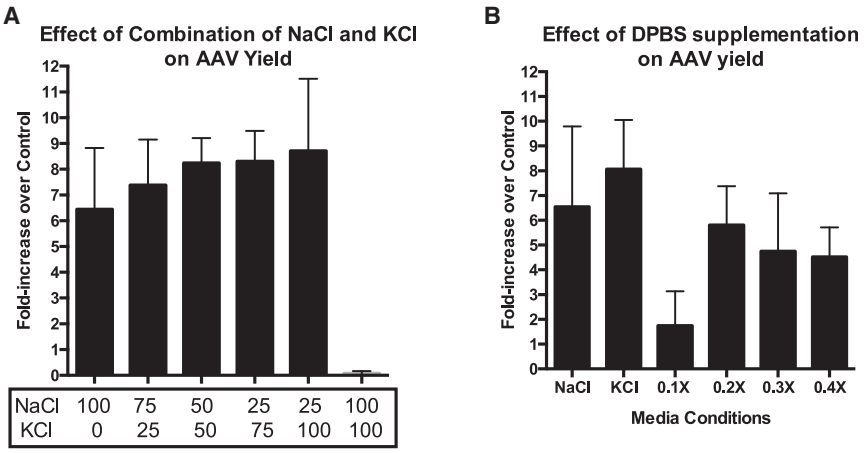

Figure 2. Effect of salt combination on AAV vector titers

(A) Combination of NaCl and KCl. Cells were infected with rHSVs in the presence of NaCl or KCl at their optimal concentration (60 and 40 mM, respectively) or with various combinations of both. For each condition, NaCl and KCl concentrations are expressed as percentage of optimal concentration such as 60 mM NaCl is 100% NaCl and 40 mM KCl is 100% KCl. Total vector genomes were obtained in cell crude lysates and expressed for each condition as the fold change over the untreated cells (control), which did not receive any salt supplementation and were set at a value of 1. The experimental run was repeated three times with each condition performed once. The figure shows the average of the three biological replicates and their standard deviation (n = 3). (B) Effect of Dulbecco's phosphate-buffered saline (DPBS) on AAV vector yield. Cells were infected with rHSVs in the presence of various concentrations of DPBS and compared to cells treated with KCl or NaCl or untreated (no salt added). DPBS concentration is expressed as a factor from 1× DPBS so that 0.2× represents a 5-fold dilution of DPBS in the cell media, and so forth (10× DPBS was used to conduct these experiments to reduce the volume of DPBS to be added to each condition). Total vector genomes were obtained in cell crude lysates and expressed for each condition as the fold change over the untreated cells (control), which were set at a value of 1. The experimental run was repeated three times with each condition performed once, except for 0.1× and 0.4× (n = 2). The figure shows the average of the biological replicates and their standard deviation.

treated and non-treated cells were similarly transduced with rHSV-GFP, resulting in more than 80% of the cells infected. KCl- and NaCl-treated cells showed approximately 10% more cells transduced. It is not excluded that salt treatment may result in an increase in GFP expression, which would result in a more sensitive detection of transduced cells.

The effect of salt supplementation is time-sensitive

In our previous study, we had noted that NaCl could be supplemented up to 6 h post-infection and maintain the same increase in effect on AAV vector production.[12] In the present study, we further delineated the precise time window within which the salt supplementation was optimal to achieve an AAV vector yield increase. In the first set of experiments, the salt supplementation was delayed by several hours after the rHSV inoculation started (or production time 0 h). We found that adding KCl at 8 to up to 10 h post-infection could still sustain 80%–90% of the AAV vector increase (Figure 4A, open circles). However, further delays (12–18 h) resulted in a further limited yield increase (30%–70% of the optimal condition). No significant effect of the salt was observed when added after 20 h post-infection. In the next set of experiments, supplementation was carried out at the time of HSV inoculation (time 0 h), but the salt was removed at various time points by media exchange (Figure 4A, filled squares). We observed that removal of the salt after 8 to up to 12 h post-infection significantly impacted the salt-mediated effect on AAV vector titers. Removal at or after 14 h sustained about 40%–50% of the optimal salt effect. The maximal effect was observed when the salt was left up to the harvest time point or 48 h. In Figure 4B, KCl was added at 8 h post-infection and left for 2–6 h. Consistent with the previous finding, the longest duration of exposure (5 and 6 h) resulted in the largest effect (70%–80% of the optimal condition). The results were confirmed in the last experiment (Figure 4C) where KCl supplementation was initiated at 8, 10, 12, or 14 h post-infection and removed after 4–6 h. Again, we found that >70% of the salt-mediated increase was maintained if the salt was left for 6 h during the production time window of 8–14 h post-infection (Figure 4C). Lastly, we assessed whether adding the salt in advance of the infection would alter the effect observed. Cells were pre-treated with NaCl or KCl 20–24 h before the infection was initiated and harvested for AAV vector titer assessment 48 h post-infection. Data presented in Figure 4D show that the presence of salt up to 24 h prior to infection did not impact the overall increase in AAV vector titers.

Altogether, these observations led us to conclude that the salt must be present at a minimum between 8 and 14 h post-infection to ensure a significant AAV rate increase, and that the first 24 h post-infection were critical in mediating the maximal effect observed. It also confirmed that our original protocol, with the salt added at the time of infection, was the most optimal, as well as the most suitable and cost-effective, in terms of the manufacturing procedure. Our data clearly indicated that the salt mechanism was temporal and transient and suggested that the effect appeared highly correlated with the kinetics of the virus production cycle and/or the cell cycle.

Change in the osmolality of AAV production media does not replicate NaCl- and KCl-induced AAV vector titer increase

Because both NaCl and KCl mediate significant changes in osmolality in a concentration-based manner, it was of interest to assess whether the AAV vector production yield increase was triggered by or at a specific osmolality value, and whether osmolality alone could be the reason for an NaCl- and KCl-mediated effect on AAV vector production. To test this hypothesis, we first assessed whether glucose, a commonly used additive in cell media, could reproduce the effect observed with KCl and NaCl. Glucose was chosen due to the fact that several studies reported the use of sucrose to mediate osmotic shock to increase mAb production, and that glucose, similar to

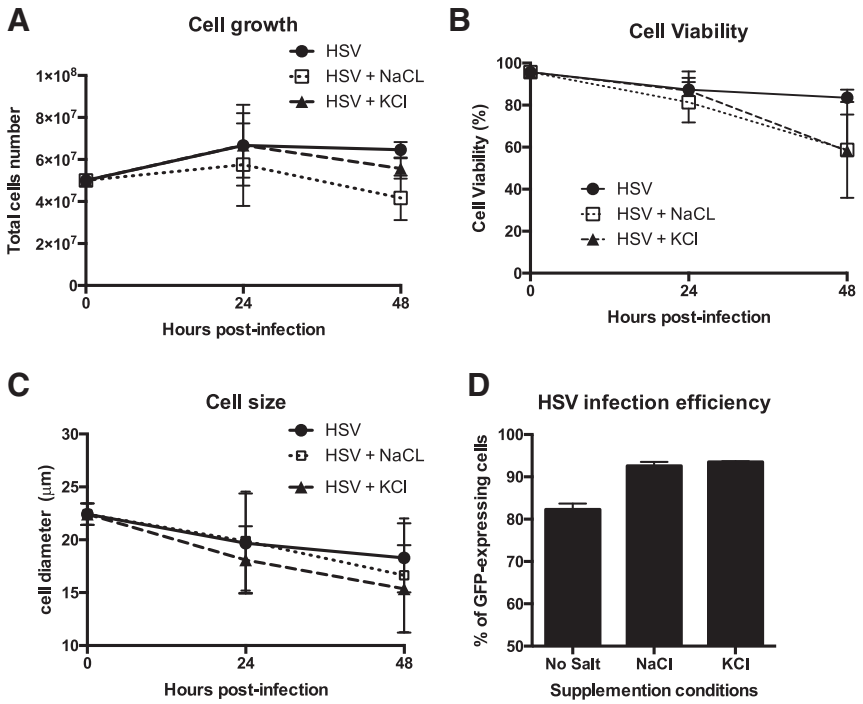

Figure 3. Effect of NaCl and KCl on cell growth, size, and viability and rHSV infection efficiency

(A–C) Cell parameters. Cells were infected in the presence or absence of KCl (40 mM) and NaCl (60 mM) and a small aliquot of cells was taken from each condition at three different time points during the production cycle, namely at the infection start (0 h), after 24 h post-infection (p.i.), and at harvest (48 h p.i.). Cell number (A), viability (B), and size (C) were assessed in duplicate in each experiment using an automated cell counter. The figure shows the averages and standard deviations from three independent experiments. (D) HSV transduction efficiency. Cells were infected with rHSV-GFP only, in the presence or absence of KCl (40 mM) or NaCl (60 mM). Cells were harvested after 8 hr and immediately submitted to flow cytometry to determine the percentage of GFP-expressing live cells. One experiment conducted with each condition performed in triplicate, except for the negative control consisting in non-infected cells. Figure 3D shows the average of the technical replicates.

sucrose, affects the medium osmolality. Glucose was added at concentrations ranging from 10 to 140 mM, and AAV vector yields were assessed in a similar fashion than previously described. Interestingly, as shown in Figure 5A, we observed an up to 3.5-fold increase in AAV vector titers when glucose was supplemented between 70 and 80 mM. However, when directly compared to KCl and NaCl in paired experiments, the glucose-mediated AAV vector increase was consistently significantly lower than the ones reported with NaCl or KCl (Figure 5B). Furthermore, and unlike with NaCl and KCl, the increase in the transducing unit titers was either similar to or lower than that of the vector genomes (Figures 5A and 5B). These data suggested that the effect of glucose, although measurable, may not act in the same ways as do KCl and NaCl.

Next, we measured the production media osmolality values in the presence of our various supplements, namely NaCl, KCl, DPBS, and glucose. As shown in Figure 5C, the osmolality value increased linearly with the concentration of NaCl, KCl, DPBS, and glucose, ranging between 300 and 400 mOsm/kg, well above the non-supplemented culture media, which we determined was 277.44 ± 1.67 mOsm/kg (vendor's specifications of 275–290 mOsm/kg). The average osmolality value at each of the compound's optimal concentration was different for each, with NaCl and KCl showing the highest values at 381 and 349, respectively, compared to 344 for 0.2× DPBS and 326 mOsm/kg for glucose at 80 mM. Alternatively, comparable osmolality values with DPBS (0.4× and 0.5×) or glucose (90–100 mM) would lead to a lower AAV vector increase, as described earlier. For a more accurate measure, we also measured the osmolality of the various production media at the time of infection and supplementation, and during the course of the production cycle (Figure 5D). We noted that the osmolality values for glucose and KCl were very similar and slightly, but not significantly, lower than the ones obtained with NaCl, and that the osmolality increased over time. Altogether, these data strongly suggested that the effect of NaCl and KCl, although correlating in part with an increase in osmolality of the production media, could not be entirely reproduced by other compounds, such as DPBS or glucose, used at the same osmolality.

Lastly, we studied the combination of KCl and glucose and tested various concentration combinations as shown in Figure 5E. Similar to what was observed for NaCl and KCl combinations, no further increases were observed when glucose and KCl were combined. However, and also similar to what was obtained previously, an upward trend was noted with increasing concentrations of KCl in the combination, suggesting that KCl alone was a powerful attribute to promote AAV vector titer increase.

Salt effect may be specific to the HSV platform

Lastly, we tested whether NaCl or KCl could improve AAV yield when produced by transfection (Figure 6). NaCl or KCl was supplemented to the production medium at the time of transfection and AAV9-GFP yields assessed. For a fair comparison, the same AAV-GFP genome was present in either plasmid pTRUF5 or HSV-GFP. AAV yields obtained by transfection were affected negatively by the salt supplementation in most cases. To rule out that the salt would impact the transfection efficiency, we tried various time points of supplementation, either before or several hours after transfection, but with no further success. However, if transfection was combined with either of the two rHSVs, an increase was observed. Altogether, these data suggest that the salt effect may be all or in part mediated by the presence of HSV functions in the producer cells. It is possible,

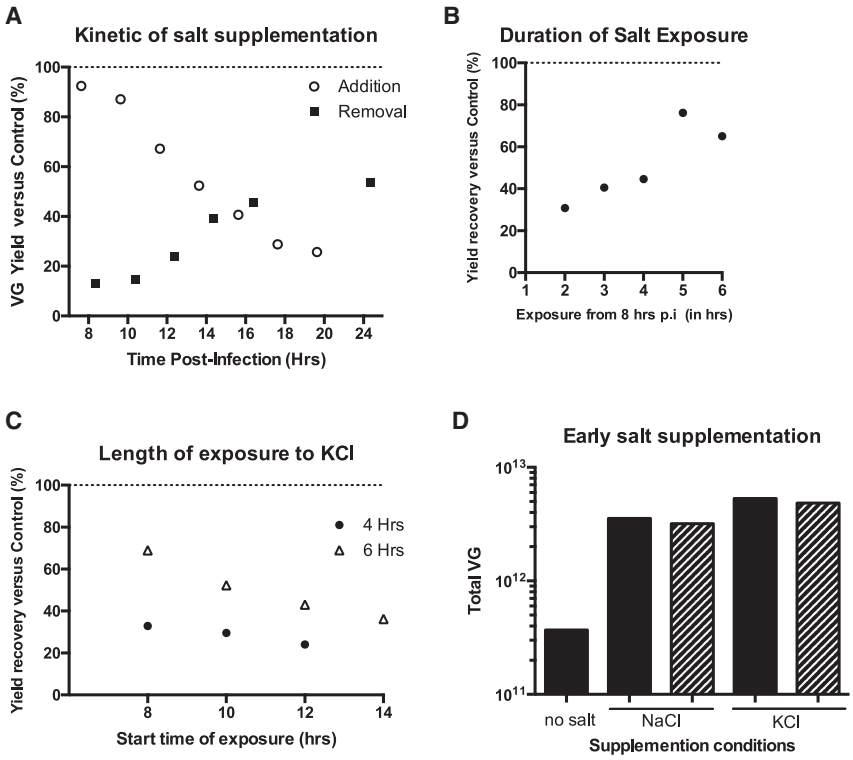

Figure 4. Timing of salt incubation for AAV vector titer increase

(A) Shortened exposure to salt. For delayed salt addition (open circles), production was initiated at time 0 h in the absence of KCl supplementation, and KCl was supplemented at 8–20 h p.i. Cells were harvested 48 h p.i., and vector genome titers were assessed in cell crude lysates. For shortened exposure, or early removal (filled squares), production was initiated at time 0 h in the presence of KCl supplementation. After 8–20 h p.i., the media were removed and replaced with fresh media and incubated for the remaining time of the production cycle. Vector genome yields obtained for each time point are expressed as a percentage of the yield obtained in the control condition when KCl is supplemented at the time of co-infection and left for the duration of the production cycle and set as 100%. Delayed addition: one representative experiment for time points 0–16 hr p.i. and one representative experiment for time points 14–20 h. Early removal: one representative experiment. (B) Time-controlled exposure to KCl from 8 h p.i. Cells were infected with rHSVs at time 0 h. In tested conditions, KCl was added to the infected cells 8 h p.i. and left from 2 to 6 h prior to medium replacement. Cells were incubated for the remaining time for a total of 48 h from the time of infection with rHSVs. Vector genome yields obtained for each time point are expressed as a percentage of the yield obtained for the control condition when KCl is supplemented at the time of co-infection and left for the duration of the production cycle (100%). One experiment was performed. (C) Time-controlled exposure to salt. In these experiments, KCl was added and removed at specific time points to create a specific length of exposure to the treatment. Exposure at 4 (filled circles) or 6 (open triangles) h were tested starting at 8, 10, 12, or 14 h p.i. with rHSVs. After 4 or 6 h of exposure, media were replaced with non-supplemented media and cells were incubated for the remaining time for a total of 48 h from the time of infection with rHSVs. Vector genome yields obtained for each time point are expressed as a percentage of the yield obtained in the control condition when KCl is supplemented at the time of co-infection and left for the duration of the production cycle (100%). One experiment was performed. (D) Salt pre-treatment. Cells were exposed to either 60 mM NaCl or 40 mM KCl for 20–24 h before infection with rHSVs and incubated for 48 h p.i., in parallel to fresh cells exposed to NaCl or KCl at the time of infection (control for treated conditions). Pre-treated conditions (striped columns) were compared to cells supplemented with salt at the time of infection (filled columns) or cells infected without salt supplementation (filled column). Total vector genomes were measured in the cell crude lysates. One representative experiment is shown out of three independent experiments, one replicate for each condition.

however, that transfection could benefit from salt supplementation in conditions that we have not identified in this work.

Conclusions

Collectively, these results convincingly demonstrated that the increase in AAV titer resulted from the addition of NaCl or KCl in a specific, temporal, and non-synergistic manner. Upon using the optimal concentration of NaCl or KCl in the production media, the average volumetric yield of AAV in cell lysates was approximately 28×10^{14} vg/L, based on small (50 mL) and large (3 L) scales or approximately $2–8 \times 10^{5}$ vg/cell, which is in line with our previously published data. Perhaps more importantly, the increase in particle yield correlated with an increase in AAV particle potency, resulting in lower vector genome-to-transducing unit ratios, suggesting that the particles produced were of improved quality.

Furthermore, although the addition of salt did affect, as expected, the osmolality of the medium within a similar range for NaCl, KCl, DPBS, or glucose, we could not find a solid and significant correlation between the osmolality value and the increase in AAV vector yield, and the overall effect of glucose, although not neglectable, was less than that observed with NaCl, KCl, or DPBS.

This work presents a novel and unique aspect of AAV biology that significantly impacted the way we produce our vector stocks, and it warrants further in-depth studies to elucidate the molecular mechanisms and pathways that are responsible for this high yield increase. This work illustrates that much is yet to discover in AAV interaction with the cell and the helper virus used to produce it, and those significant improvements can arise from such studies. The impact of any rate increase of AAV production can be critical to support industrial and commercial scales and facilitate AAV viral vectors use for human gene therapy.

DISCUSSION

Increasing production yields will be paramount to ensure the success of AAV vectors at commercial levels, both to sustain the demands for a one-time high dose or multi-dose product, but also to mitigate the cost per dose in favor of affordability. While almost any production

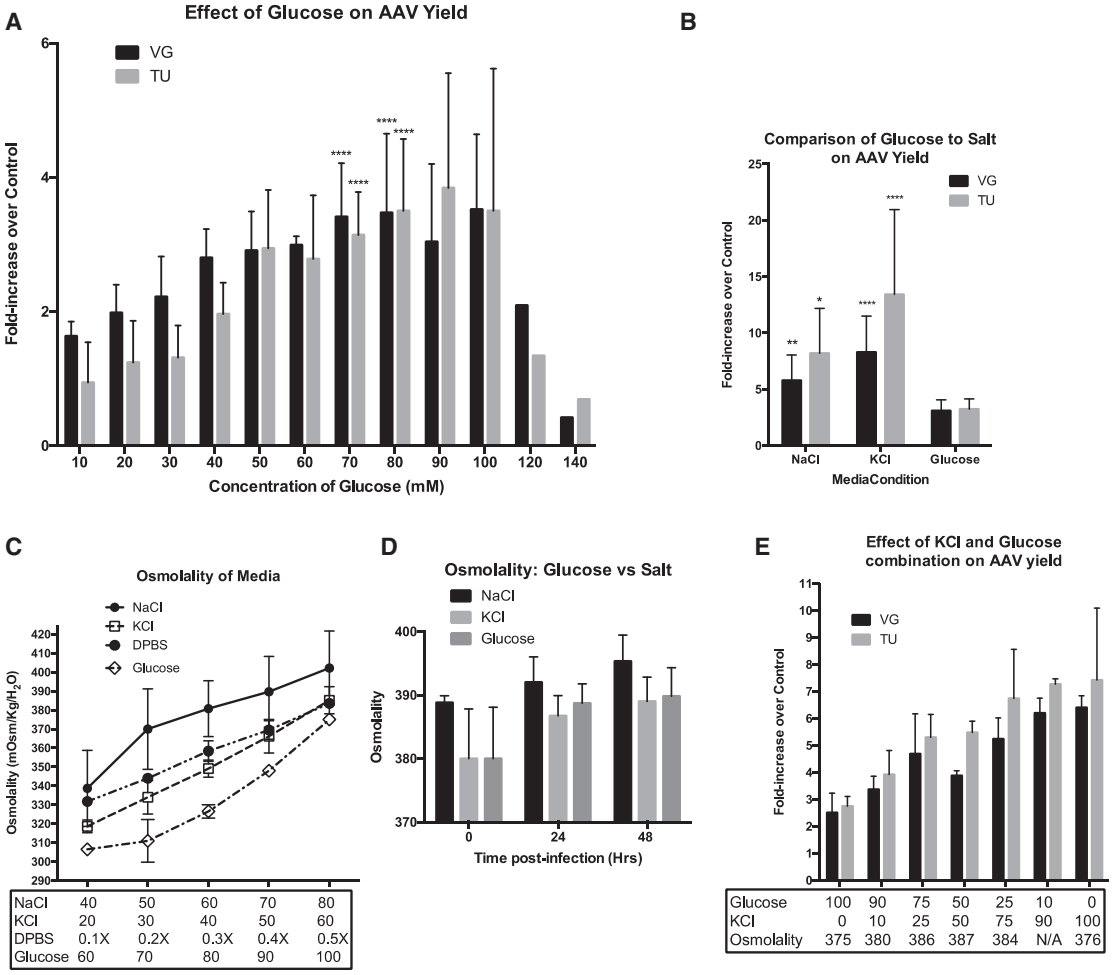

Figure 5. Effect of osmolality on AAV vector titers

(A) Effect of glucose on AAV vector yield. Cells were infected in the presence of various concentrations of glucose and compared to non-treated cells. Total vector genomes and transducing units were assessed in cell crude lysates and compared to the value obtained for the non-treated cells within each experiment (ratio or fold-change value). (A) Average and standard deviation of these ratios from n independent experiments: glucose 10, 40, and 50 mM, n = 3; 20, 30, and 60 mM, n = 4; 70 mM, n = 8; 80 and 90 mM, n = 9; 100 mM, n = 5; 120 and 140 mM, n = 1). (B) Comparison of glucose to KCl and NaCl. Experiments were conducted as above using KCl, NaCl, and glucose (70 mM) at their optimal concentration. The fold-increase ratio of total vector genome and transducing units for each condition versus their paired control (non-treated) were calculated for each experiment and averaged from n independent experiments (KCl, glucose, n = 7; NaCl, n = 4). *$p < 0.05$. **$p < 0.001$. ****$p < 0.0001$. (C) Media osmolality mediated by salt and glucose. Osmolality value upon supplementation of culture media with various concentrations (mM or fold dilution) of NaCl, KCl, DPBS, and glucose. (D) Osmolality during AAV vector production. Osmolality values of the production media were measured at time of infection (0 h, n = 5), 24 hr p.i. (n = 3), and at harvest (48 h p.i., n = 4). The figure shows the averages and standard deviation from five independent experiments or as noted for each time point. (E) Combination of KCl and glucose. For each condition, KCl and glucose concentrations are expressed as percentage of their optimal concentration (40 and 80 mM, respectively) (n = 3 independent experiments).

scale can be expanded by multiplying the number of bioreactors and/or the volume per batch, any improvement that specifically increases the yield of vector produced per each producer cell is highly valuable. However, a good understanding of AAV biology and its interaction with the host cell and helper virus is key to achieve this goal, yet many aspects of it need to be discovered. It is well accepted that the production of rAAV is short to reproduce the yields obtained with the WT virus. Typical rAAV-specific yields often range around 1×10^4 AAV vg/cell, far lower than the $>1 \times 10^6$ vg produced during the course of the WT-AAV infection.[2]

In a somewhat unexpected manner, we found that the simple addition of NaCl to the production medium could increase AAV vector yield by more than 1 log when using the HSV infection system.[12] This was the first time such a finding was reported for AAV vectors. In the current study, we further documented that KCl, but no other salts tested, was able to achieve a similar or higher rate increase than NaCl. We demonstrated that the increase was not due to an increase in cell number, cell viability, or size, strongly supporting the fact that the specific yield, or the production of AAV particles per cell, was significantly improved.

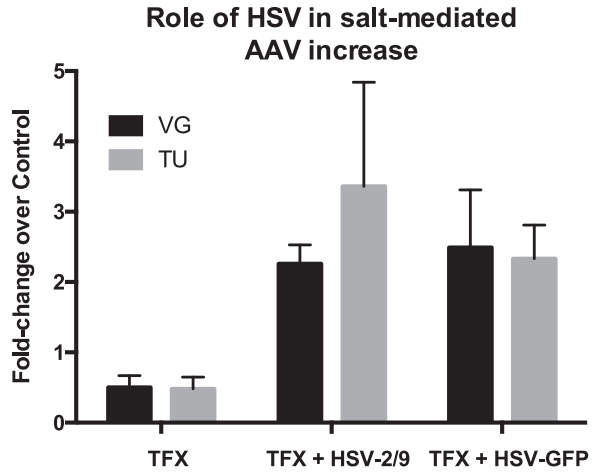

Figure 6. Effect of salt on AAV vector yields when produced by transfection

AAV9-GFP was produced by transfection with two plasmids, pTRUF5 and pDG9-KanR (n = 6), or by transfection of the AAV genome (pTRUF5) in combination with the HSV helper (HSV-2/9) (n = 4), or by transfection of the helper plasmid pMA-P5-Rep2/cap9 and infection with the HSV-GOI (HSV-GFP) (n = 5), in the presence or absence of salt (NaCl or KCl). Ratios over the matching non-treated conditions are shown as average of n independent experiments.

Another critical finding was that the infectious titers of the vector produced in the presence of salt consistently showed a superior increase than did the particle numbers, which is rather an unusual observation. Because we provided some preliminary pieces of evidence that the effect mediated by NaCl or KCl was not observed when AAV vector was produced by transfection, unless in the presence of HSV, we hypothesize that the unique effect of salt in our production platform somehow triggers a cascade of events involving HSV genes and possibly cellular genes that creates a very favorable cellular environment for efficient and timely AAV replication and packaging, resulting in vector genome and infectious titer increases. Perhaps the kinetics and dynamics of viral and cellular events more similarly resemble those triggered during a productive cycle of the WT virus. Whether the AAV vector yield increase is the result of specific cellular or HSV functions modulated upon the presence of NaCl and/or KCl remains to be identified. The improved potency could also be the result of our relatively short production cycle. As we reported before, the peak of production of rAAV when using the HSV system in our suspension system is between 24 and 48 h. A recent study by Wilson and colleagues[13] showed that prolonged production cycles beyond 3–5 days could result in significantly reduced vector infectivity, linked to deamidation of capsid residues. Another interesting finding was that the effect of salt was temporal and transient and likely fast-acting within an 8- to 16-h post-infection time window. Because both HSV and AAV Reps interfere with the cell cycle, it is possible that the time window identified above is directly linked to a cell cycle phase induced by the HSV and/or the rAAV replication in the producer cells. During this time frame we would expect that most of the infected cell population would have reached the G_1 or the S phase, which would also be in accordance with AAV and HSV active role in modulation of the cell cycle to their benefit.[14,15]

A logical deduction from our data would be that NaCl and KCl ion channels and/or ion transporters would be likely involved in mediating this effect. Located in the cell membrane, they regulate the flux of ions between the intracellular and extracellular environment.[16] They are diverse and involved in multiple physiological cellular responses. The cellular effects mediated by high salt concentration have been described in a variety of models from cancer,[17] inflammation, cell proliferation, and gene expression, and in some cases led to the discovery of salt-induced enzymes such as salt-induced kinase.[18] However, the underlying mechanisms remain to be fully identified. In our model in particular, we can extrapolate that the concentrations of KCl and NaCl in the extracellular environment were approximately 5 and 110 mM, respectively, based on the formula for standard culture media such DMEM (note that the formula of the Expi growth medium has not been disclosed). Upon supplementing the salts, these concentrations increased to approximately 45 mM for KCl and 180–200 mM for NaCl (including the addition of the HSV stocks). In mammalian cells, the intracellular concentration of KCl (~139 mM) is about 10-fold higher than that of NaCl (~12 mM),[19] while the extracellular concentrations are lower for KCl and higher for NaCl. For KCl, the extracellular concentration was increased by approximately 8-fold in our experimental runs, and for NaCl by approximately 2-fold. The mechanisms by which these ions interfere with the Na^+ and K^+ ions pumps and channels are not known at this time and warrant further studies.

Our work also explored the impact of increased osmolality during the production phase of rAAV. While similar studies addressing virus production are still very limited, osmotic shock, created by a sudden increase in the medium osmolality, is a commonly used strategy to increase production of mAbs.[4,20] Most of these studies have shown that a very general effect of an increase in osmolality (typically above 300 mOsm) results in a sharp decrease in cell growth, whether using Chinese hamster ovary (CHO) kidney cells,[20–24] hybridoma cells,[25,26] or HEK293 cells.[27–29] Since mAbs are typically produced from established mammalian cell lines that produce mAbs continuously, the overall production yield relies simultaneously on the total cell numbers, after a multi-day growth phase, and the specific yield, or protein production per cell. To overcome the negative effect of high osmolality on cell growth, a common mAb production strategy relies on a bi-phasic batch culture approach, with a growth phase under iso-osmotic conditions, followed by a production phase at high osmolality.[20–23,26,28] In our model, growth and production are temporally separated: cells are amplified before the production phase, counted, and set at a defined density to ensure proper rHSV MOIs, and cell growth is limited from that point, which in a way could resemble the bi-phasic production strategy used for mAbs. It is also widely accepted that the effect of osmotic shock is cell-specific, and the osmolality range varies depending on the cell line and the media used, as we previously showed for BHK and HEK293 cells.[9,12] Typically, osmolality values utilized for CHO cells ranged between 425 and 522 mOsm[20–23] and for hybridoma cells between 370 and 400 mOsm approximately.[25,26] Despite these similarities, there are a series of notable differences between our model and mAb production. The first and foremost difference is that the increase in osmolality was not, at least solely, responsible for the increase in AAV

production. While the underlying mechanisms supporting mAb increased production have not been thoroughly studied, and various, sometimes contradicting, explanations have been proposed, it is well accepted that the osmolality change is the direct cause for the increase in production. Evidence for this conclusion is that various compounds, such as glucose, sucrose, mannitol, or sorbitol, can reproduce the effect observed with NaCl.[4,20,27,30] Second, the yield increase described in our study was notably higher than the rate of increase reported for mAb production, with most studies reporting a relatively moderate mAb rate increase between 2- and 3-fold.[20,23,26] This is likely the result of critical differences between mAbs and rAAV production: mAb production relies essentially on transcription and translation in cells that are selected for this performance; on the contrary, rAAV production involves genome rescue and replication, gene expression, and genome packaging, all steps heavily controlled both by cellular and helper virus pathways. In our model, even a modest increase in AAV replication could exponentially impact AAV gene expression and packaging. In an attempt to elude the molecular mechanisms supporting the mAb rate increase, some studies have reported changes in the metabolism of the producer cells, but no clear consensus could be drawn to support a common pathway.[20,31,32]

To our knowledge, the use of osmotic shock, or the use of salts, to increase viral production is not as common as for mAbs. Studies using HEK293 cells to produce Ad have led to opposite conclusions than ours, namely reporting a decrease in yield in hyperosmotic media.[27,28] One study by Shen et al.,[29] reported a significant increase in Ad production upon modulating the osmolality value between the growth and production phases. In these studies, however, 293 cells were adapted to a higher osmolality (370 mOsm) by passaging and prior to the production of Ad. Importantly, production needed to be performed under isotonic conditions.[33,34] The hypothesis suggested by the authors was that the pre-conditioning of the cells in high osmolality medium induced favorable physiological conditions for the virus production. This is in sharp contrast with our model since we clearly showed that the presence of salt was required during the production phase. This would suggest that the effect of osmolality and/or salt addition is different for each virus and/or cell line combination utilized. This result is consistent with our finding that Ad-based production of rAAV (transfection) was not impacted by the salt.

In conclusion, our study provides unique and important new insights on AAV biology and highlights the importance of studying the influence of biological parameters on AAV production cycle, as they are key to enable the establishment of successful production protocols. Our long-term goal is to identify the molecular elements and/or pathways that enable the effect of NaCl and KCl on rAAV production. We think that once these mechanisms are identified, it may be possible to translate the findings to optimize rAAV production yields from other production methods, specifically transient transfection.

MATERIALS AND METHODS

Cell lines

Expi293F cells (Thermo Scientific, Waltham, MA, USA) were maintained in Expi293 Expression medium (Thermo Fisher Scientific) and grown in shaker incubators (Multitron, Infors HT) at 5% CO_2, 37°C, 125 rpm, and 75% humidity. Cell concentration was maintained between approximately 5×10^5 and 5×10^6 cells/mL during passaging. Cell counts, viability, and size were assessed using a Countess II automated cell counter (Thermo Fisher Scientific). For all production experiments a cell viability superior to 90% was required. V27 cells (a gift from Dr. Knipe, Harvard, MA, USA) and C12 cells (originally obtained from Dr. P. Johnson, Children's Hospital of Philadelphia, Philadelphia, PA, USA) were maintained in DMEM supplemented with 5% FBS and 50 μg/mL Geneticin (Sigma, St. Louis, MO, USA).

Plasmids

Plasmid pTR-UF5, pDG-UF9-KanR, and pMA-P5-Rep2Cap9 were previously described in detail in Adamson-Small et al.[9] pTR-UF5 contains an AAV2-ITR vector genome cassette for the expression of humanized green fluorescent protein (hGFP) under the cytomegalovirus (CMV) promoter, identical to the rAAV genome present in rHSV-GFP. Plasmid pDG-UF9-KanR contains the AAV2 Rep and AAV9 capsid open reading frames (ORFs under the MMTV and AAV2 P19/P40 promoters and the Ad5 helper genes as described in Grimm et al.[6] Plasmid pMA-P5-Rep2Cap9 contains the endogenous AAV2 Rep ORF with p5, p19, and p40 promoters and an AAV9 capsid sequence.

rHSV production

rHSV-GFP was created by introducing the complete recombinant vector genome of rAAV2-UF5 within the genome of HSV-1 deletion mutant d27.1. AAV2-UF5 contains the WT-AAV2 ITRs, the CMV enhancer/promoter, and the hGFP coding sequence (described in detail in Adamson-Small et al.[9]). Helper rHSV-AAV9 contained the entire expression cassette of AAV2 Rep and AAV9 Cap under their respective endogenous promoters, as described in Adamson-Small et al.[9] Production of rHSV-GFP and rHSV-AAV9 stocks was performed by infecting V27 cells in 10-layer CellSTACKs (Corning Life Sciences) at an MOI of 0.15 plaque-forming unit (PFU)/cell. At 72–96 h post-infection, cells were lysed in 0.6 M NaCl (Lonza, Allendale, NJ, USA), harvested, and cell debris was removed by centrifugation. The rHSV-containing cell lysate was concentrated by tangential flow filtration using TangenX SIUS (300 kDa) (catalog no. XP300L01L, TangenX, Shrewsbury, MA, USA). rHSV stocks were stored frozen at −80°C in 5% glycerol (Thermo Fisher Scientific).

rAAV viral vector production and purification

Small-scale AAV production

Expi293F cells were seeded in 125-mL Corning Erlenmeyer cell culture flasks with vented Cap (Corning Life Sciences, MilliporeSigma) at 1×10^6 cell/mL in 25- or 50-mL final culture volumes. Cells were co-infected within 1 h at an MOI of 2:4 with rHSV-GFP and rHSV-AAV9, respectively. For media supplementation experiments, the inoculum containing rHSV, cell media, and the defined salts or glucose concentrations as described in the text were mixed and immediately added to the cell-containing flasks. Supplementing reagents were NaCl solution (5 M, AccuGene, Lonza), KCl solution (2 M, Thermo Fisher Scientific), DPBS with calcium and magnesium solution (10× DPBS, Corning Life Sciences, Cellgro), K_2HPO_4 solution (1

M, MilliporeSigma, St. Louis, MO, USA), KH_2PO_4 solution (1 M, MilliporeSigma, St. Louis, MO, USA), Na_2HPO_4 solution (0.5 M, MilliporeSigma, St Louis, MO, USA), $ZnCl_2$ solution (0.1 M, MilliporeSigma, St Louis, MO, USA), $MgCl_2$ solution (1 M, Thermo Fisher Scientific), $CaCl_2$ solution (1 M, Sigma, St. Louis, MO, USA), $MnCl_2$ solution (1 M, MilliporeSigma, St. Louis, MO, USA), Na_2SeO_3 powder (MilliporeSigma, St Louis, MO, USA), iron(III) nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$ powder (MilliporeSigma, St. Louis, MO), and glucose (200 g/L solution, Thermo Fisher Scientific). All salt solutions were diluted in Expi293 expression medium. For solid salts, stock solutions were prepared in cell media and sterile filtered (100 mM $Fe(NO_3)_3 \cdot 9H_2O$; 3 mM Na_2SeO_3).

Two days after infection, cells were spun down (2,000 rpm, 4°C, 20 min, Sorvall ST-40R, Thermo Scientific), rinsed in 1× DPBS (HyClone), and spun down again. When required, the supernatants were sampled for pH and osmolality and stored at 4°C. Vector genome and infectious titers were assessed in cell crude lysates prepared after a series of three freeze-thaws in 2.5- or 5-mL lysis buffer (50 mM Tris, 150 mM NaCl [pH 8.5]), Benzonase (EMD Millipore, Billerica, MA, USA) digestion (100 U/mL, 30 min, 37°C), and clarification (3,700 rpm, 20 min, 4°C, Sorvall ST-40R). Crude lysates were stored at −80°C for the duration of the study.

For the timing experiments, cells were either co-infected as described above, in the presence or absence of KCl, and the medium was exchanged after the desired number of hours and replaced with fresh medium without KCl and incubated for the remaining of the production period; alternatively, cells were co-infected in the absence of KCl and KCl was added at a later time, left for the duration for the production period, or removed after the desired number of hours and replaced with fresh medium without KCl. For the early supplementation experiments, cells were passaged slightly above 1×10^6 cells/mL, 20–24 h prior to infection, and 60 mM NaCl or 40 mM KCl was added to the growth medium. On the day of infection cells were counted and concentrations adjusted to 1×10^6 cells/mL with fresh medium. When fresh medium was added, the appropriate amount of NaCl or KCl was added to obtain 60 mM NaCl or 40 mM KCl in the presence of both rHSVs.

Unless otherwise specified, all experiments were performed as biological replicates. Infection runs were repeated independently (n = number of independent experiments, shown in the figure legends) on different days, with various lots of HSVs or cell culture medium. Within each run, all of the conditions were processed and assessed concurrently to provide paired data with the non-treated conditions. Values obtained for each biological replicate were averaged and the standard deviation was calculated. We think that this is the most representative approach to verify the statistical relevance of a biological effect by capturing the biological parameters' variability across experiments.

Large-scale production and purification

Large-scale production was performed in 5-L Corning Erlenmeyer cell culture (MilliporeSigma) flasks with a 3-L production volume and a total of 3×10^9 cells. Infection and supplementation were performed similarly to the small-scale production but grown at 100 rpm. AAV purification was previously described in Adamson-Small et al.[9] Briefly, cells were lysed, and lysates were digested with Benzonase (EMD Millipore, Billerica, MA, USA) (crude lysates), clarified by protein flocculation, and followed by centrifugation. Viral particles were purified using a two-step chromatography approach: first, a cation-exchange step on SP Sepharose fast flow (Cytiva/GE Healthcare, Marlborough, MA, USA) was followed by an affinity-based capture using POROS CaptureSelect AAV9 affinity resin (Thermo Fisher Scientific). The final eluate was formulated in 1× DPBS by dialysis using 10K Slide-A-Lyzer dialysis cassettes (3–12 mL, Thermo Fisher Scientific). Final concentrated AAV stocks were stored at −80°C.

AAV production by transient transfection

Small-scale transient transfection (50 mL) was performed in Expi293 cells (5×10^7 cells in 125-mL shaker flasks) using PEI Max 40K (Polysciences, Warrington, PA, USA). Plasmids were transfected at equimolar amounts (50 μg total) and the salts added at the time of transfection and left for 72 h before harvest. For infection and transfection combinations, cells were first transfected for 4 h and then infected with rHSV and salts. Cells were harvested after 2 days for analysis.

rAAV-specific assays

Vector genome titer

Vector genome titers were obtained by quantitative real-time PCR as previously described[12] using primers UF5-3F (5′-CCAGGTCCAC TTCGCATATT-3′) and UF5-3R (5′-GCGTGCAATCCATCTTG TTC-3′) and plasmid pTR-UF5 for the standard curve.

Transduction assay

Ad5-infected C12 cells (MOI of 20) were transduced in 96-well plates by serial dilutions of the rAAV-containing samples. After 42–48 h, GFP-expressing cells were visually counted under a microscope, and UV light and titer were calculated based on sample dilution and volume.

HSV-specific assays

Plaque assay

rHSV infectious titers were assessed by a traditional plaque assay. V27 cells were seeded at 1.2×10^5 cells per well in 24-well plates (Corning Life Sciences). Serial dilutions of the rHSV stocks were added to the cells and incubated for 1.5–2 h. The inoculate was removed and DMEM containing 0.8% agar (LMT; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was poured in each well. Plaques were counted by microscopic examination at 72 h post-infection.

Flow cytometry

Cell sorting for GFP expression was performed and analyzed by the Cytometry Core of the Interdisciplinary Center for Biotechnology Research, University of Florida, using a BD LSRFortessa (BD Biosciences, San Jose, CA, USA) and BD FACSDiva 8.0.2 software. Uninfected Expi293F cells were used as a negative control to set the gate

for GPF fluorescence detection. The total numbers of GFP-positive cells in live gated cell populations were measured in each condition.

pH and osmolality

Each sample was brought to room temperature before analyzing pH and osmolality.

pH was measured using an Accumet model AR 15 pH meter with an Orion PerpHect ROSS combination electrode. The pH meter was calibrated with US standard buffers (pH 4 and 7), and the slope was determined to be between 95% and 102% for successful calibration. Osmolality was measured using a Precision Touch Micro OSMETTE 6002 osmometer was used to determine the osmolality of samples according to the manufacturer's protocol. The osmometer was calibrated with calibration buffers in triplicates (100, 500, and 1,500 mOsm/kg) during each test and before analyzing the samples.

Statistical analysis

GraphPad Prism software (GraphPad, La Jolla, CA, USA) was used to analyze differences between groups utilizing a one-way ANOVA followed by Dunnett's post-test for group comparison. p values <0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.02.015.

ACKNOWLEDGMENTS

We would like to thank Dr. Chen Ling (University of Florida, Gainesville, FL, USA) and the State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University (Shanghai, China) for allowing C.Y. to join the N.C. laboratory as part of the visitor exchange program. At the time of this work was completed, C.Y. was also a student affiliated with the State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University (Shanghai, China). We would like to thank Dr. Scott A. Rivkees (Department of Pediatrics, University of Florida, USA) for providing Departmental Seed Funds to N.C. to support part of this research work.

AUTHOR CONTRIBUTIONS

N.C. designed the original study, performed data analysis, and prepared the manuscript. N.C., C.Y., P.C., and R.B. conducted the production experiments and transduction assays. P.D.T. performed large-scale purification (with M.P.) and the osmolality studies. E.J.J. and T.C. conducted PCR, pH, and various quality control assays. B.J.B. helped with the constructive review of the manuscript.

DECLARATION OF INTERESTS

N.C. and B.J.B. are inventors on patent applications U1202.70016US02 and U1202.70060US00 related to this research. B.J.B. is an inventor of intellectual property owned by the Johns Hopkins University and the University of Florida related to the HSV system and has AAV patents licensed to various biopharmaceutical companies. The remaining authors declare no competing interests.

REFERENCES

1. Keeler, A.M., and Flotte, T.R. (2019). Recombinant adeno-associated virus gene therapy in light of Luxturna (and Zolgensma and Glybera): where are we, and how did we get here? Annu. Rev. Virol. *6*, 601–621.
2. Clément, N., and Grieger, J.C. (2016). Manufacturing of recombinant adeno-associated viral vectors for clinical trials. Mol. Ther. Methods Clin. Dev. *3*, 16002.
3. Salganik, M., Hirsch, M.L., and Samulski, R.J. (2015). Adeno-associated virus as a mammalian DNA vector. Microbiol. Spectr. *3*.
4. Li, F., Vijayasankaran, N., Shen, A.Y., Kiss, R., and Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. MAbs *2*, 466–479.
5. Xiao, X., Li, J., and Samulski, R.J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J. Virol. *72*, 2224–2232.
6. Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J.A. (1998). Novel tools for production and purification of recombinant adenoassociated virus vectors. Hum. Gene Ther. *9*, 2745–2760.
7. Muzyczka, N. (1992). Use of adeno-associated virus as a general transduction vector for mammalian cells. Curr. Top. Microbiol. Immunol. *158*, 97–129.
8. Clément, N., Knop, D.R., and Byrne, B.J. (2009). Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies. Hum. Gene Ther. *20*, 796–806.
9. Adamson-Small, L., Potter, M., Falk, D.J., Cleaver, B., Byrne, B.J., and Clément, N. (2016). A scalable method for the production of high-titer and high-quality adeno-associated type 9 vectors using the HSV platform. Mol. Ther. Methods Clin. Dev. *3*, 16031.
10. Alazard-Dany, N., Nicolas, A., Ploquin, A., Strasser, R., Greco, A., Epstein, A.L., Fraefel, C., and Salvetti, A. (2009). Definition of herpes simplex virus type 1 helper activities for adeno-associated virus early replication events. PLoS Pathog. *5*, e1000340.
11. Kotin, R.M., and Snyder, R.O. (2017). Manufacturing clinical grade recombinant adeno-associated virus using invertebrate cell lines. Hum. Gene Ther. *28*, 350–360.
12. Adamson-Small, L., Potter, M., Byrne, B.J., and Clément, N. (2017). Sodium chloride enhances recombinant adeno-associated virus production in a serum-free suspension manufacturing platform using the herpes simplex virus system. Hum. Gene Ther. Methods *28*, 1–14.
13. Giles, A.R., Sims, J.J., Turner, K.B., Govindasamy, L., Alvira, M.R., Lock, M., and Wilson, J.M. (2018). Deamidation of amino acids on the surface of adeno-associated virus capsids leads to charge heterogeneity and altered vector function. Mol. Ther. *26*, 2848–2862.
14. Franzoso, F.D., Seyffert, M., Vogel, R., Yakimovich, A., de Andrade Pereira, B., Meier, A.F., Sutter, S.O., Tobler, K., Vogt, B., Greber, U.F., et al. (2017). Cell cycle-dependent expression of adeno-associated virus 2 (AAV2) Rep in coinfections with herpes simplex virus 1 (HSV-1) gives rise to a mosaic of cells replicating either AAV2 or HSV-1. J. Virol. *91*, e00357-1.
15. Flemington, E.K. (2001). Herpesvirus lytic replication and the cell cycle: arresting new developments. J. Virol. *75*, 4475–4481.
16. Brown, B.M., Nguyen, H.M., and Wulff, H. (2019). Recent advances in our understanding of the structure and function of more unusual cation channels. F1000Res. *8*, 8.
17. Amara, S., and Tiriveedhi, V. (2017). Inflammatory role of high salt level in tumor microenvironment (Review). Int. J. Oncol. *50*, 1477–1481.
18. Amara, S., Majors, C., Roy, B., Hill, S., Rose, K.L., Myles, E.L., and Tiriveedhi, V. (2017). Critical role of SIK3 in mediating high salt and IL-17 synergy leading to breast cancer cell proliferation. PLoS ONE *12*, e0180097.
19. Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., and Darnell J. (2000). Intracellular ion environment and membrane electric potential. In Molecular Cell Biology, Fourth Edition (W.H. Freeman), section 15.4.
20. Nasseri, S.S., Ghaffari, N., Braasch, K., Jardon, M.A., Butler, M., Kennard, M., et al. (2014). Increased CHO cell fed-batch monoclonal antibody production using the autophagy inhibitor 3-MA or gradually increasing osmolality. Biochem. Eng. J. *91*, 37–45.

21. Han, Y.K., Koo, T.Y., and Lee, G.M. (2009). Enhanced interferon-beta production by CHO cells through elevated osmolality and reduced culture temperature. Biotechnol. Prog. *25*, 1440–1447.

22. Ozturk, S.S., and Palsson, B.O. (1991). Effect of medium osmolarity on hybridoma growth, metabolism, and antibody production. Biotechnol. Bioeng. *37*, 989–993.

23. Kim, M.S., Kim, N.S., Sung, Y.H., and Lee, G.M. (2002). Biphasic culture strategy based on hyperosmotic pressure for improved humanized antibody production in Chinese hamster ovary cell culture. In Vitro Cell. Dev. Biol. Anim. *38*, 314–319.

24. Zhu, M.M., Goyal, A., Rank, D.L., Gupta, S.K., Vanden Boom, T., and Lee, S.S. (2005). Effects of elevated pCO_2 and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study. Biotechnol. Prog. *21*, 70–77.

25. Cherlet, M., and Marc, A. (1999). Hybridoma cell behaviour in continuous culture under hyperosmotic stress. Cytotechnology *29*, 71–84.

26. El-Enshasy, H.A., Adawy, H.H.E., and Yang, S.T. (2007). Effects of different osmotic pressure of the cultivation media on hybridoma cell growth and monoclonal antibodies production kinetics in batch culture. Biotechnology *6*, 202–209.

27. Nadeau, I., Garnier, A., Côté, J., Massie, B., Chavarie, C., and Kamen, A. (1996). Improvement of recombinant protein production with the human adenovirus/293S expression system using fed-batch strategies. Biotechnol. Bioeng. *51*, 613–623.

28. Ferreira, T.B., Ferreira, A.L., Carrondo, M.J., and Alves, P.M. (2005). Two different serum-free media and osmolality effect upon human 293 cell growth and adenovirus production. Biotechnol. Lett. *27*, 1809–1813.

29. Shen, C.F., and Kamen, A. (2012). Hyperosmotic pressure on HEK 293 cells during the growth phase, but not the production phase, improves adenovirus production. J. Biotechnol. *157*, 228–236.

30. Sharfstein, S.T., Shen, D., Kiehl, T.R., and Zhou, R. (2007). Molecular responses to osmotic shock. In Systems Biology Cell Engineering, *5* (Springer), pp. 213–236.

31. Sun, Z., Zhou, R., Liang, S., McNeeley, K.M., and Sharfstein, S.T. (2004). Hyperosmotic stress in murine hybridoma cells: effects on antibody transcription, translation, posttranslational processing, and the cell cycle. Biotechnol. Prog. *20*, 576–589.

32. Shen, D., Kiehl, T.R., Khattak, S.F., Li, Z.J., He, A., Kayne, P.S., Patel, V., Neuhaus, I.M., and Sharfstein, S.T. (2010). Transcriptomic responses to sodium chloride-induced osmotic stress: a study of industrial fed-batch CHO cell cultures. Biotechnol. Prog. *26*, 1104–1115.

33. Petiot, E., Cuperlovic-Culf, M., Shen, C.F., and Kamen, A. (2015). Influence of HEK293 metabolism on the production of viral vectors and vaccine. Vaccine *33*, 5974–5981.

34. Shen, C.F., Voyer, R., Tom, R., and Kamen, A. (2010). Reassessing culture media and critical metabolites that affect adenovirus production. Biotechnol. Prog. *26*, 200–207.