

# How Endotoxin Contamination Can Affect Gene and Cell Therapies

Gene therapy is revolutionising the way we treat human diseases. Any technique that modifies a person's genes to treat or cure a disease is considered a form of gene therapy. This can occur via several possible mechanisms. A disease-causing version of a gene may be inactivated or replaced with a healthy version. Alternatively, a new gene may be introduced to combat a disease. Gene therapy products work by introducing genetic material into the nucleus of the cell. To introduce the genetic material, scientists need a delivery system that can transport the gene, nuclease, or short hairpin RNA (shRNA) to the nucleus of a human cell. The vehicle that carries this genetic material is known as a vector.<sup>1</sup>

A wide variety of vectors are available for gene therapy and can be categorised into viral and non-viral types. Viral vectors serve as the current delivery system used in FDA-approved gene therapies, while non-viral techniques are being studied as a safe and effective way to deliver genetic material to cells for therapeutic effect.<sup>1</sup> In addition, viral vectors have in comparison to non-viral vectors demonstrated 10 times to 1000 times higher efficacy of gene transfer. We should note; however, that the high safety levels and low production costs for non-viral vector-based gene therapies are highly attractive features that continue to be considered in the development of future medicines.<sup>5</sup>

The two most common vectors are plasmids and viruses. A plasmid is a small, extrachromosomal DNA molecule within a cell that is physically separated from chromosomal DNA and can replicate independently. They are most often found as small circular, double-stranded DNA molecules in bacteria, but are sometimes present in archaea and eukaryotic organisms. When found in nature, plasmids often carry genes that benefit the survival of the organism and can provide distinctive advantages, such as a strong resistance to antibiotics. While chromosomes are large and contain all the essential genetic

information for living under "normal conditions", plasmids are usually very small and contain only additional genes that may be useful in certain situations of stress and adversity or during states of disease.<sup>2</sup> On the other hand, the genes packaged by viral vectors can be integrated into the host cells' genomes and permanently expressed. Some types of viruses insert their genome into the host's cytoplasm, but do not actually enter the cell, while others have been found to penetrate the cell membrane disguised as protein molecules by which entry into the cell is easily accomplished. There are two main types of viral infections that can occur. One is known as a lytic infection and the other a lysogenic infection. Shortly after inserting its DNA, viruses of the lytic cycle quickly produce more viruses then burst from the cell to continue infecting more and more cells. Lysogenic viruses integrate their DNA into the DNA of the host cell and may live in the body for many years before responding to a trigger. The virus reproduces as the cell does and does not inflict any harm to the harbouring host until it is triggered in some way. Once triggered, the virus releases the DNA from that of the host and employs it to create new viruses.<sup>3</sup>

The first viral vector used in gene therapy was based on adenovirus, which is a virus that causes the common cold, as well as other respiratory, intestinal, and ocular infections in humans.<sup>1</sup> The genetic material of the adenovirus is carried in the form of double-stranded DNA. When introduced into a host cell, this genetic material remains transient in the nucleus, thus allowing it to be freely transcribed just like any other gene. Unfortunately, the adenovirus has been found to trigger strong, potentially dangerous, immune reactions in patients, so research using these types of viruses in gene therapy has been ongoing.<sup>3</sup> Other viral vectors such as retrovirus and herpes simplex virus have also been used.

Like all human therapeutics, it is critical for gene therapy products to be free of endotoxin contamination. Endotoxin, also known as lipopolysaccharide or LPS, is a

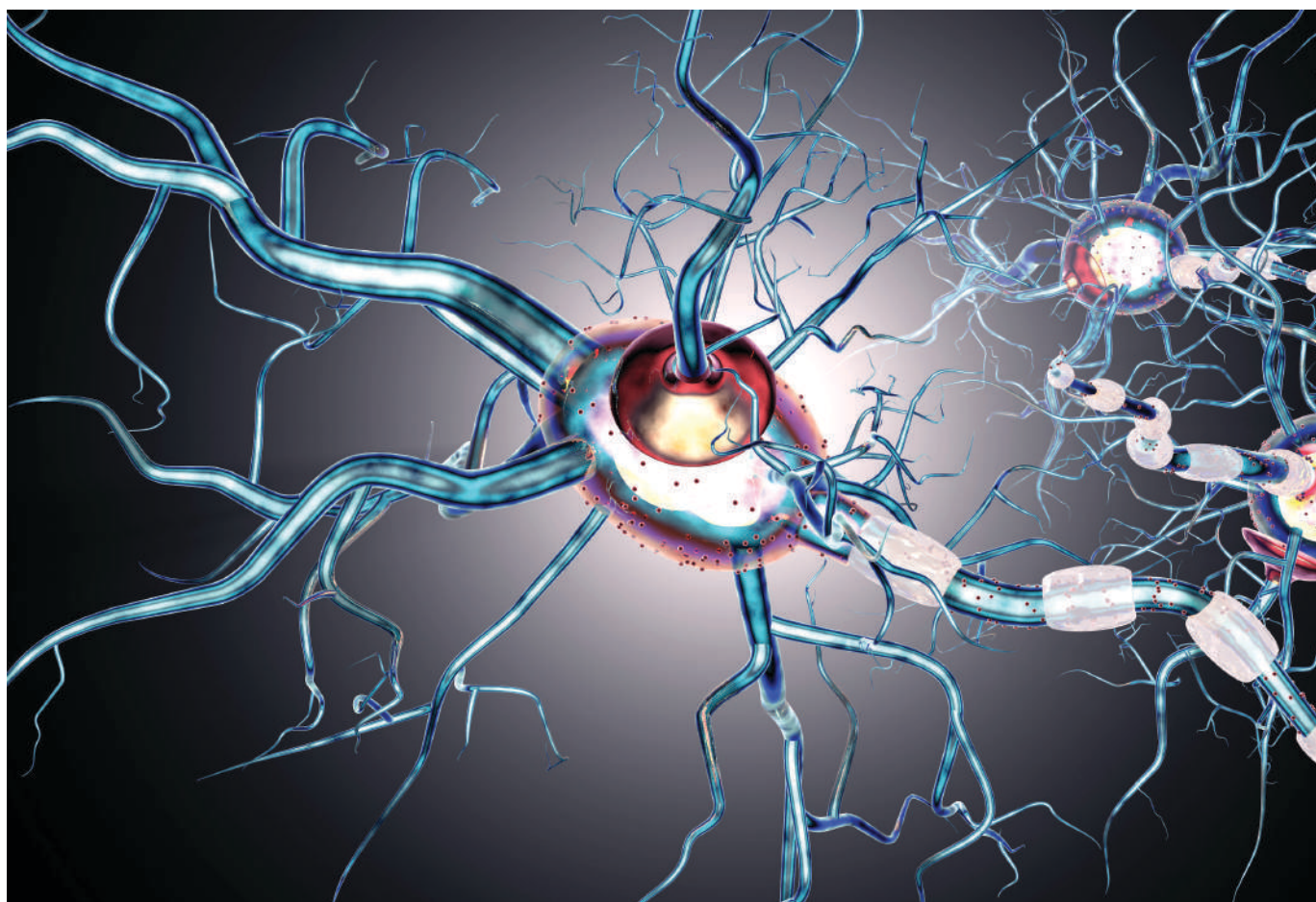
component of the outer cell membrane of Gram-negative bacteria. It is an extremely potent pyrogen, with even miniscule exposure leading to dangerous fever or even sepsis. Furthermore, endotoxin is highly heat-resistant and thus difficult to remove through traditional means.

According to FDA guidelines, all intravenously injected pharmaceutical products must contain below 5 endotoxin units per kg of body weight. Endotoxin is highly ubiquitous, with lab environments being no exception, and so it is crucial for gene therapy products to be tested for endotoxin contamination prior to use in human subjects.

A 2019 paper published in *Molecular Therapy – Methods & Clinical Development* tested a new method to remove endotoxin contamination from recombinant adeno-associated virus (rAAV) stocks, a common vector for gene therapy. rAAV is prepared using plasmid DNA isolated from *E. coli* bacteria, which is a frequently a source of endotoxin contamination.<sup>8</sup>

The authors used the LAL assay to quantify endotoxin levels. One of the challenges with decontaminating rAAV stocks is that any residual detergents could not only induce toxicity, but also interfere with the LAL assay reagents, leading to false negatives. This is due to the masking effect, wherein an LPS molecule becomes surrounded by detergent molecules and thus shielded from interacting with the LAL reagents. However, the authors were able to keep detergent levels below critical levels in their decontaminated stocks, allowing for accurate endotoxin readouts using LAL.<sup>8</sup>

This study highlights the importance of thorough decontamination of gene therapy products, as well as the necessity of stringent buffer-exchange washing in order to remove residual detergent. As the popularity of gene therapy increases, it will remain crucial for scientists to be aware of the potential dangers of endotoxin contamination and the need to avoid false negatives caused by detergent carryover.<sup>8</sup>



As with therapeutics based on gene therapy, the possible contamination of cell therapy products is also something to be considered. Cellular therapy products include cellular immunotherapies, cancer vaccines, and other types of both autologous and allogeneic cells for certain therapeutic indications, including hematopoietic stem cells and adult and embryonic stem cells. Although gene therapy involves the transfer of genetic material into the appropriate cells by way of a carrier or vector, cell therapy is the transfer of cells having a relevant and necessary function into a patient.<sup>4,6</sup>

When culturing any kind of cells in a laboratory environment, avoiding contamination is always a chief concern. Biological contaminants are often the focus of such efforts, and they also can be the most straightforward to detect and avoid.

For instance, most bacterial or fungal contamination can be visually detected in the cell culture media and prevented using antibiotic treatments. Other biological contaminants, such as mycoplasma or other cell lines, are more difficult to detect, but can still be monitored via commercially available testing kits.

In contrast to biological contamination, chemical contamination receives relatively little attention and is more difficult to detect and avoid. Among the most insidious chemical contaminants are endotoxins. Potential sources of endotoxin contamination include water, cell culture media, sera, glassware, and plasticware. As mentioned with the gene therapy products, endotoxins found in cell therapy are highly resistant to both autoclaving and irradiation, meaning they can be present even in the absence of viable bacteria. Their high hydrophobicity also gives them a strong affinity for plasticware and unlike live bacteria, endotoxins cannot be observed visually in cell culture media. Furthermore, endotoxins cannot be depleted with antibiotics, instead requiring specialised endotoxin removal solutions.

By taking steps to avoid endotoxin-induced cell culture problems, researchers can be more confident in experimental results. Various methods have been suggested to aid in keeping cell cultures and their resulting therapies free of endotoxin contamination. Among those are: utilisation of high-purity water and low-endotoxin FBS, as well as incorporating the use of plasticware that is certified to

be endotoxin-free.<sup>7</sup> However, in addition to the use of purified raw materials and reagents, establishing procedures for strong aseptic technique and sterilisation will be vital in reducing the chances of endotoxin contamination.

Aseptic technique is one of the core skills for any biology researcher. Preventing contamination of cell cultures is necessary to avoid experimental artifacts and potential cell death. Additionally, contamination in an animal research context could lead to infections or death.

Most biological contaminants can be avoided using standard sterilisation reagents, such as bleach or ethanol. However, endotoxin is highly stable and can persist even in the absence of viable bacteria. Thus, it is crucial for QC technicians to maintain rigorous standard operating procedures for aseptic technique.

One example of an aseptic technique relevant to endotoxin is the practice of changing one's gloves regularly. An inexperienced cell culture technician may assume that frequently spraying their gloves with ethanol is sufficient to maintain sterility. However, ethanol can





leave endotoxin contamination behind, so it is important to set standards for how frequently users should change their gloves.

Endotoxin contamination can greatly disrupt an *in vitro* experiment, particularly those involving immune cells. Macrophages show increased IL-6 secretion in response to endotoxins, while T cells show increased proliferation and lymphokine production.

Non-immune cells can also be subject to dysregulation by endotoxins. Though endotoxins are classically viewed to act through the CD14 receptor, cells lacking this receptor can still show strong responses to endotoxin contamination. For instance, one study reported that cardiac myocytes experience contractile dysfunction upon exposure to endotoxins. Other studies have reported altered protein production in CHO cells and altered clonal efficiency in ureteral epithelial cells.

Additionally, the sensitivity of different cell lines to endotoxin contamination is highly variable. Some cell lines show dysregulation at less than 1 ng/mL of endotoxin, whereas others require much higher concentrations of up to 5000 ng/mL. It has also been theorised that cell lines grown for many years in culture (such as HeLa and CHO cells) may have been naturally selected for endotoxin resistance over time. Based on this, it is difficult to determine a universal safe threshold for endotoxin contamination.

When working with cells in culture, it is crucial to purchase low-endotoxin products when available. However, since

endotoxin contamination can arise after opening reagents, or be transferred from glassware/plasticware, it is also important to perform regular endotoxin testing.

For both gene therapy and cell therapy products, the Limulus amoebocyte lysate (LAL) assay is a method that offers a cost-effective and highly sensitive option to quantify endotoxin levels. The assay relies on proteins extracted from the blood of horseshoe crabs and in the presence of endotoxin, these proteins undergo a clotting reaction which can be quantified to give a highly accurate readout of endotoxin levels. This assay continues to play a crucial part in maintaining the safety of our gene and cell therapy treatments, particularly when used during large-scale productions or crucial *in vitro* experiments.

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