REVIEW

The Role of Zinc in Antiviral Immunity

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ABSTRACT

Zinc is an essential trace element that is crucial for growth, development, and the maintenance of immune function. Its influence reaches all organs and cell types, representing an integral component of approximately 10% of the human proteome, and encompassing hundreds of key enzymes and transcription factors. Zinc deficiency is strikingly common, affecting up to a quarter of the population in developing countries, but also affecting distinct populations in the developed world as a result of lifestyle, age, and disease-mediated factors. Consequently, zinc status is a critical factor that can influence antiviral immunity, particularly as zinc-deficient populations are often most at risk of acquiring viral infections such as HIV or hepatitis C virus. This review summarizes current basic science and clinical evidence examining zinc as a direct antiviral, as well as a stimulant of antiviral immunity. An abundance of evidence has accumulated over the past 50 y to demonstrate the antiviral activity of zinc against a variety of viruses, and via numerous mechanisms. The therapeutic use of zinc for viral infections such as herpes simplex virus and the common cold has stemmed from these findings; however, there remains much to be learned regarding the antiviral mechanisms and clinical benefit of zinc supplementation as a preventative and therapeutic treatment for viral infections. *Adv Nutr* 2019;10:696–710.

Keywords: zinc, virus, metallothionein, antiviral, immunity, zinc deficiency, zinc supplementation

Introduction

Zinc deficiency was first recognized by Prasad et al. >50 y ago in a malnourished group of individuals presenting with hepatosplenomegaly, dwarfism, hypogonadism, and an elevated risk of infection (1). Unbeknownst to Dr. Prasad and his colleagues at the time, their discovery would highlight the importance of zinc as an integral component of human physiology, and inspire decades of zinc research. It is now understood that zinc is the second-most abundant trace metal in the human body after iron, and an essential component of protein structure and function. Importantly, zinc is a structural constituent of ∼750 zinc-finger transcription factors (2) enabling gene transcription, and is a catalytic component of approximately 2000 enzymes, encompassing all 6 classes (hydrolase, transferase, oxido-reductase, ligase, lyase, and isomerase) (3). Hence, zinc is biologically essential for cellular processes, including growth and development, as well as DNA synthesis and RNA transcription (4).

The global prevalence of zinc deficiency is estimated to range from ∼17% to 20% (5, 6), with the vast majority occurring in developing countries of Africa and Asia. Although significantly less common in high-income nations, zinc deficiency occurs most frequently in the elderly, vegans/vegetarians, and individuals with chronic disease such as liver cirrhosis (7) or inflammatory bowel disease (8). Importantly, zinc deficiency results in a compromised immune system, as evidenced by thymic atrophy, lymphopenia, and defective lymphocyte responses in animal studies (9). These data underscore the importance of zinc nutrition, particularly in underdeveloped countries where the risk of infection is heightened because of poor sanitation, public health, and vaccination strategies (5).

This review focuses on the role of zinc as an essential micronutrient that is required to mount an effective antiviral response. Although zinc possesses direct antiviral properties (e.g. influenza), it is also critical in generating both innate and acquired (humoral) antiviral responses. To complicate matters, zinc is an integral component of many viral enzymes, proteases, and polymerases, highlighting the importance of regulating cellular and systemic zinc distribution to inhibit viral replication and dissemination.

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Abbreviations used: EV, *epidermodysplasia verruciformis*; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HPV, human papilloma virus; HSV, herpes simplex virus; IRF, IFN regulatory factor; ISG, interferon stimulated gene; MT, metallothionein isoforms; MTF1, metal-responsive transcription factor; PRR, pattern recognition receptor; RdRp, RNA-dependent RNA polymerase; RT, reverse transcriptase; SOCS, suppressors of cytokine signaling; TLR, Toll-like receptor; ZIP, Zrt- and Irt-like proteins.

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Current Status of Knowledge

Zinc homeostasis and viral infection

Systemic and intracellular zinc are tightly regulated, such that free zinc ions (Zn^{2+}) represent a minimal fraction of total cellular zinc (~0.0001%) (10–12). The vast majority of zinc remains bound to zinc-binding proteins such as serum albumin or intracellular metallothionein proteins, where it can be transferred to zinc-binding enzymes and transcription factors as necessary. Zinc transport is principally mediated by 2 groups of proteins: the ZnT [solute-linked carrier 30 (*SLC30A*)] family, which is responsible for efflux of zinc outside the cell or influx into organelles, and the ZIP [Zrt- and Irt-like proteins (*SLC39A*)] family of proteins, which performs the opposite role, transporting zinc into the cytoplasm from extracellular sources or cellular organelles (13). The >30 human proteins responsible for zinc homeostasis collectively ensure that zinc does not become toxic in the case of dietary excess, nor limited in the case of dietary insufficiency. Of course, this balance cannot be maintained indefinitely, and may result in zinc-induced copper deficiency if consumed in excess (14), and severe zinc deficiency if it is lacking in the diet (1).

Sequestration and toxic accumulation of metals are well-documented antibacterial immune responses. Calprotectin is a prime example, binding and sequestering extracellular calcium and zinc, thus preventing bacterial and fungal overgrowth (15). Conversely, toxic endosomal zinc accumulation can inhibit intracellular *Mycobacterium* growth in macrophages (16). Unfortunately, these mechanisms are not well described in the case of viral infections, perhaps because of a lack of efficacy. Calprotectin, for example, has no proven antiviral role, nor is it significantly upregulated in response to viral gastroenteritis (17). This absence of a zinc-mediated antiviral response may reflect the "parasitic" nature of viral infection, hijacking host machinery to self-replicate. Changes in intracellular zinc concentrations necessary to inhibit viral replication may also prove toxic to eukaryotic cells for the same reason.

Although antiviral modulation of zinc homeostasis in humans remains unproven, papilloma viruses have evolved mechanisms to alter zinc homeostasis to favor viral replication and persistence (18). The human papilloma virus (HPV) E5 protein can interact with the zinc transporter ZnT-1 in complex with EVER2, thus stimulating nuclear accumulation of zinc (19). The ZnT-1:EVER2 complex responsible for zinc export from the nucleus is inhibited by HPV E5, subsequently increasing both nuclear zinc and the activation of AP1 (20), a transcription factor required for HPV genome expression. Interestingly, homozygous mutations in either EVER1 or EVER2 result in a rare condition termed *epidermodysplasia verruciformis* (EV). EV patients are particularly susceptible to HPV strains 5 and 8, which significantly increases the risk of developing nonmelanoma skin cancers. HPV strains 5 and 8 lack expression of the E5 protein, which may explain *1*) their limited replication in the normal population because of their inability to control zinc homeostasis, and *2*) the susceptibility of EV patients to strains 5 and 8 from the loss of EVER protein function, favoring HPV replication. Interestingly, HPV E5 genes have co-evolved with the major HPV oncogenes, E6 and E7, and indicate the potential involvement of E5 in carcinogenesis (21, 22). Clinical trials using both oral and topical zinc have proven effective for the treatment of viral warts, and will be reviewed in a later section.

Metallothioneins, zinc homeostasis, and antiviral activity. Metallothioneins are small, cysteine-rich proteins capable of binding divalent cations such as zinc and copper. As vessels for much of the labile intracellular zinc pool, metallothioneins possess numerous functions through their ability to bind and release metals from their thiol groups. These include storage and transfer of zinc ions and heavy metal detoxification, as well as involvement in oxidative stress, apoptosis, and immune responses (23). Humans express 4 metallothionein isoforms (MT1–4), including the ubiquitously expressed MT1 and MT2 genes (MT1A, B, E, F, G, H, I, J, L, M, X, MT2A), as well as MT3 and MT4 whose expression is limited, and function remains poorly understood (24). Importantly, MT1 and 2 gene expression is extremely responsive to zinc, and therefore serves as an ideal indicator of an individual's zinc status (25). Upon taking a zinc supplement, for example, an increase in protein-bound zinc in the bloodstream is internalized by cells in various tissues and organs through the ZIP transporters. In response to increased intracellular zinc, the metal-responsive transcription factor (MTF1) becomes active, and binds the metal responsive element in metallothionein gene promoters to upregulate their transcription (26). Although there are additional stimuli that influence metallothionein expression, this primarily occurs in a zinc-dependent fashion. Oxidative stress, for example, induces zinc release from metallothioneins as a mechanism to reduce reactive oxygen species generated by mitochondrial dysfunction or viral infection (26). Zinc released from metallothioneins binds MTF1 to stimulate additional metallothionein expression.

It should be noted that metallothioneins, although highly responsive to zinc, have long been classified as interferon stimulated genes (ISGs) (27). IFNs are immunostimulatory cytokines secreted from infected cells and nearby immune cells that induce the expression of hundreds of antiviral genes. They possess diverse roles including chemoattraction, immune cell activation, and direct antiviral activity. In response to IFNs, we suggest that there are 2 mechanisms of metallothionein induction. Most ISGs possess binding sites for STAT- or IFN regulatory factor (IRF) transcription factor-mediated expression, as is the case for MT1X and MT2A (28, 29). Other metallothioneins such as MT1F and MT1G do not possess known IFN regulatory regions in their promoters, but are instead more sensitive to zinc (28). IFNs stimulate an influx of zinc into the target cell, as is the case with some inflammatory cytokines such as IL-6, which in turn drives metallothionein expression.

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Because metallothioneins possess such a diverse functional repertoire, their specific roles during viral infection remain undefined. However, both in vitro and in vivo studies have made it abundantly clear that metallothioneins are induced by viruses. The mechanisms often remain undefined; however, metallothionein expression has been attributed to zinc influx or redistribution (19, 28), by viral means, cytokine exposure, or oxidative stress (30). Metallothionein upregulation has been observed in response to measles virus (31), influenza (31, 32), HIV (33), hepatitis C virus (HCV) (34), and coxsackie virus (35), among others. In the case of HIV, zinc appears to be the key driver of metallothionein expression to favor viral persistence. HIV-infected monocytes demonstrate a significant increase in both MT1 gene expression as well as intracellular zinc (33). Elevated intracellular zinc increases monocyte resistance to apoptosis via inhibition of caspase 3 activation [as has been reported previously (36)], thus providing a reservoir for HIV replication. The role of metallothioneins remains unclear in this study; however, they have been described as negative regulators of apoptosis, albeit not through direct caspase 3 inhibition (37). Zinc and metallothioneins also facilitate human cytomegalovirus (HCMV) replication by activating the immediate-early HCMV promoter (38, 39). Kanekiyo et al. demonstrated that both zinc and metallothionein overexpression increased NF-κB binding in the HCMV promoter. Because no complex was detected between metallothionein and NF-κB, it was suggested that metallothioneins served as a zinc donor necessary for NF-κB binding. In addition, as NF-κB transcription factors are known potent activators of HIV and HSV replication, and several other viruses (40), metallothioneins may be proviral. Zinc has also been reported to inhibit NF-κB in numerous studies (41–43). Despite these contrasting data, Kim et al. have bridged these inconsistencies, demonstrating that MT2A can serve as a sink for excess zinc (44), thus limiting its proximity to NF-κB and favoring NF-κB-mediated transcription.

In the case of HCV infection, metallothioneins possess an antiviral role. Using a pan-metallothionein siRNA to knockdown all MT1 and 2 genes, we demonstrated both an increase in HCV replication and a decrease in intracellular zinc content in vitro (34). Interestingly, although $ZnSO_4$ can reduce HCV replication, this effect was ablated when metallothionein genes were knocked down. These data suggest that metallothioneins are either *1*) directly antiviral, potentially by sequestering zinc away from viral metalloproteins such as HCV NS5A (45), or *2*) indirectly antiviral by acting as zinc chaperones and facilitating antiviral signaling. Further, metallothioneins possess antiviral properties against other viruses as well, as demonstrated in an antiviral screen of 380 human ISGs performed by Schoggins et al. (46). Overexpression of multiple members of the MT1 family inhibited replication of flaviviruses including yellow fever virus and HCV, as well as the alphavirus Venezuelan equine encephalitis virus. This effect was not observed in West Nile virus, and Chikungunya virus. These data indicate that metallothioneins, like many ISGs, are selectively antiviral, perhaps reflecting specific viral zinc requirements during replication. This is particularly evident for HIV, which demonstrated an increase in viral replication as a result of metallothionein overexpression in the Schoggins et al. ISG screen (46), validating previous works (33).

Zinc as an antiviral: bench to bedside and back again

Many studies have evaluated the efficacy of zinc as an antiviral agent in vitro. Unfortunately, zinc concentrations used to assess antiviral activity often far exceed physiological concentrations. Human plasma zinc, for example, ranges from approximately 10 to 18 μM (47), whereas antiviral concentrations of zinc can reach into mM concentrations (48). Intracellular zinc concentrations range from 10s to 100s of μM, but are significantly buffered by zinc-binding proteins such as metallothioneins, rendering free zinc concentrations at picomolar to low nanomolar concentrations (49, 50). The antiviral properties of zinc are certainly virus-specific, but it would appear that zinc ion availability plays a significant role in the antiviral efficacy of zinc (51). Here we describe the role of zinc as a virus-specific antiviral: both in vitro mechanistic studies, as well as human-based clinical trials using zinc supplementation. In vitro and in vivo studies are summarized in **Tables 1** and **2**, respectively.

Herpesviridae.

The effect of zinc on HSV-1 and -2 has been studied for >40 y, with in vitro studies suggesting that zinc plays an inhibitory role on almost every aspect of the viral life cycle: viral polymerase function (52), protein production and processing (53), and free virus inactivation (48, 54). Although these studies were performed >20 y ago, a more recent study using the zinc ionophore pyrithione demonstrated a reduction in HSV replication from reduced NF-κB activation by interfering with the protein ubiquitination pathway (41). Unfortunately, no recent experimental data can demonstrate with any certainty the mechanism by which zinc inhibits HSV infection. Nonetheless, in vivo studies in mice and humans have shown a significant reduction of infection and disease burden. Mouse studies performing intravaginal zinc inoculation in liquid (55) or gel (56) form both resulted in significant reductions in HSV-2 infection. Several topical zinc application studies have been performed in humans, which demonstrated a significantly reduced recurrence and duration of infection (outbreak) (57–58). The efficacy of topical application, together with in vitro results (48, 54), suggest that free zinc may indeed coat HSV virions, thus preventing infection. Further research into this molecular mechanism is warranted.

Apart from HCMV mentioned above, the effect of zinc on other members of the *Herpesviridae* family remains unknown because of a lack of clinical data. Mechanistically, zinc ions have been shown to inhibit Varicella-Zoster virus by inactivating free virus in vitro (59). Both HSV and Varicella-Zoster virus belong to the *Alphaherpesvirinae* subfamily, reflecting their genetic relatedness, and similar mechanism of inhibition.

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TABLE 1 In vitro studies assessing the antiviral efficacy of zinc[1]

Virus	Antiviral effect	Zinc	Effective dose	Reference
Coronavirus	Inhibition of RdRp template binding and elongation	PT + $Zn(OAc)_2$	2–320 μM PT + 2–500 μM Zn	(60)
Encephalomyocarditis virus	Inhibition of viral polyprotein cleavage	$ZnCl_2$	0.4–1.5 mM	(61)
	Inhibition of viral polyprotein cleavage	$ZnCl_2$	0.1–1 mM	(62)
	Inhibition of viral polyprotein tertiary structure	PT, HK	5–20 μM PT, 60–125 μM HK	(63)
	Inhibition of viral polyprotein tertiary structure	PDTC	15–125 μM PDTC	(63)
Foot and mouth disease virus	Inhibition of viral polyprotein cleavage	$ZnCl_2$, $Zn(OAc)_2$	0.1–2 mM	(64)
	Inhibition of viral RNA and procapsid synthesis	$ZnCl_2$	10–150 μM	(65)
Hepatitis C virus	Inhibition of RNA polymerase	$ZnCl_2$	60–300 μM	(66)
	Inhibition of viral replication	$ZnCl_2$, $ZnSO_4$	50–150 μM	(67)
	Metallothionein-dependent inhibition of viral replication	$ZnSO_4$	50 μM	(34)
Herpes simplex virus	Viral protein synthesis	$ZnSO_4$	N/A	(53)
	Inhibition of viral DNA polymerase	$Zn(OAc)_2$	0.1–2 mM	(52)
	Free virus inactivation	$ZnSO_4$	0.1–6 mM	(48)
	Free virus inactivation	$Zn(Glu)_2$, $Zn(Lac)_2$	1–50 mM	(54)
	Inhibition of protein ubiquitination and NF-κB activity	PT	1.2–18.9 mM	(41)
Human immunodeficiency virus	HIV protease inhibition	Not listed	0.2–2 mM	(68)
	Inhibition of viral transcription and particle production	$ZnCl_2$	70–700 μM	(69)
	Inhibition of reverse transcriptase	$ZnCl_2$	25–800 μM	(70)
Human papilloma virus	Stimulates proviral transcription factor activity, reversed by EVER2	N/A	N/A	(19)
	Inhibition of viral protein E6 and E7 synthesis stimulating apoptosis	CIZAR	500–750 μM	(71)
Respiratory syncytial virus	Reduction in viral titer and plaque count	$ZnCl_2$, $Zn(OAc)_2$, $Zn(Lac)_2$	0.01–10 mM	(72)
Rhinovirus	Inhibition of viral polyprotein cleavage	$ZnCl_2$	100–800 μM	(73, 74)
	Inhibition of viral polyprotein cleavage	$ZnCl_2$	0.1–1.2 mM	(61)
	Inhibition of viral polymerase	not listed	>0.6 μM	(75)
	Inhibition of viral polyprotein processing	PT, HK	5–20 μM PT, 60–125 μM HK	(63)
	Inhibition of viral polyprotein processing	PDTC	15–125 μM PDTC	(63)
Semliki Forest virus	Inhibition of endosomal membrane fusion	$ZnCl_2$	25–100 μM	(76)
	Inhibition of endosomal membrane fusion	$ZnCl_2$	2 mM	(77)
Sindbis virus	Inhibition of viral particle production and polyprotein cleavage	$ZnCl_2$	0.1–1.8 mM	(78)
Transmissible gastroenteritis virus	Inhibition of viral RNA and protein synthesis	$ZnCl_2$, $ZnSO_4$	10–200 μM	(79)
Vaccinia virus	Inhibition of RNA synthesis and viral yield	$ZnSO_4$	100–300 μM	(80)
	Inhibition of viral particle production and polyprotein cleavage	$ZnCl_2$	50–400 μM	(81)
	Inhibition of viral topoisomerase	Not listed	2.5 mM	(82)
Varicella-zoster virus	Free virus inactivation	$Zn(Pic)_2$, $Zn(Asp)_2$	10 μM	(59)

[1] CIZAR, zinc citrate compound; HK, hinokitiol; N/A, not applicable; PDTC, pyrrolidine-dithiocarbamate; PT, pyrithione; RdRp, RNA-dependent RNA polymerate; $Zn(Asp)_2$, zinc aspartate; $ZnCl_2$, zinc chloride; $Zn(Glu)_2$, zinc gluconate; $Zn(Lac)_2$, zinc lactate; $Zn(OAc)_2$, zinc acetate; $Zn(Pic)_2$, zinc picolinate; $ZnSO_4$, zinc sulfate.

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TABLE 2 Human clinical studies using zinc as an antiviral therapy [1]

Viral infection/condition	Antiviral/therapeutic effect	Effective dose	Treatment	Reference
Torque teno virus	Reduced viral load following stem cell transplant	600 mg $ZnSO_4$/d	Oral	(83)
Herpes simplex	Reduced duration and severity of outbreak	ZnO/glycine cream (0.3% ionic Zn)	Topical	(57)
	Reduction in outbreak recurrence	0.025% $ZnSO_4$ solution	Topical	(84)
	Reduction in outbreak recurrence	1–4% $ZnSO_4$ solution	Topical	(58)
Experimental rhinovirus	Reduced duration of illness with $Zn(Glu)_2$ only	$Zn(Glu)_2$ (13.3 mg) or $Zn(OAc)_2$ (5/11.5 mg) lozenges, every 2–3 h/d	Lozenge	(85)
Common cold	Reduced symptom severity, frequency, and duration	ZGG lozenges containing 23 mg Zn, every 2 h/d	Lozenge	(86)
	Reduced symptom severity, frequency, and duration	ZGG lozenges containing 24 mg Zn, every 2–3 h/d (Max 8)	Lozenge	(87)
	Reduced duration of symptoms	ZGG lozenges containing 13 mg Zn, every 2 h/d	Lozenge	(88)
	Reduced symptom severity and duration	$Zn(OAc)_2$ lozenges each containing 9 mg Zn, every 2 h/d	Lozenge	(89)
	Reduced symptom severity and duration	$Zn(OAc)_2$ lozenges each containing 13 mg Zn, every 2–3 h/d	Lozenge	(90)
	No effect on duration or severity	$Zn(Glu)_2$ (13.3 mg) or $Zn(OAc)_2$ (5/11.5 mg) lozenges, every 2–3 h/d	Lozenge	(85)
	Reduced symptom severity and duration	$Zn(OAc)_2$ lozenges each containing 13 mg Zn, every 2–3 h/d	Lozenge	(91)
Viral warts	Improved clearance of warts after 1–2 mo	10 mg/ kg $ZnSO_4$ to a maximum dose of 600 mg/d	Oral	(92)
	Clearance of warts based on concentration of zinc used	3 × 5 or 10% $ZnSO_4$/d	Topical	(93)
	Improved clearance of warts after 1–2 mo	10 mg/ kg $ZnSO_4$ to a maximum dose of 600 mg/d	Oral	(94)
	No benefit	10 mg/ kg $ZnSO_4$/d	Oral	(95)
	Resolution of 88% of lesions after 6 wk/3 sessions	Up to 3 intralesional injections with 2% $ZnSO_4$	Injection	(96)
Laryngeal papillomatosis	Resolution of papillomatosis (2 case studies)	10 mg/ kg $ZnSO_4$/d	Oral	(97)
HIV	Reduced infection, increased CD4 T cell count	200 mg/d $ZnSO_4$/d	Oral	(98)
	Increased CD4 T cell count	45 mg $Zn(Glu)_2$ every 8 h for 15 d, then 15 mg for 15 d	Oral	(99)
	Reduced incidence of diarrhea	10 mg elemental zinc as $ZnSO_4$/d	Oral	(100)
	No benefit	25 mg/d $ZnSO_4$/d	Oral	(101)
Chronic hepatitis C virus	Enhanced response to IFN treatment	2 × 75 mg polaprezinc/d	Oral	(102)
	No benefit to IFN treatment response	5 × 78 mg $Zn(Glu)_2$/d	Oral	(103)
	Reduced serum AST, ALT, and ferritin	3 × 75 mg polaprezinc/d	Oral	(104)
	Reduced serum ALT and Th2 cells (%)	2 × 75 mg polaprezinc/d	Oral	(105)
	Reduced incidence of HCC (albumin-dependent)	2 × 150 mg polaprezinc/d	Oral	(106)

[1] ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; ZGG, zinc gluconate/glycine; $Zn(Glu)_2$, zinc gluconate; ZnO, zinc oxide; $Zn(OAc)_2$, zinc acetate; $ZnSO_4$, zinc sulfate.

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Picornaviridae.

It was clear as early as 1974 that zinc possessed an inhibitory effect on picornovirus polyprotein processing (73). Before 1980, zinc inhibition of picornovirus proteases from human rhinovirus isolates (73, 74), encephalomyocarditis virus (62), poliovirus (61), and foot and mouth disease virus (64, 65) had all been demonstrated. More recent studies using zinc ionophores have illustrated that zinc interferes with the autocatalytic processing of the viral protease $3CD^{pro}$ into $3C^{pro}$ in the picornavirus coxsackievirus B3, thus inhibiting processing of the viral polyprotein (107). However, this was not the case for encephalomyocarditis virus, where zinc appeared to inhibit the tertiary structure within the viral polyprotein (107). Together, these data suggest that zinc may interfere with proteolytic processing of the viral polyprotein because of misfolding, or through direct actions on the viral protease $3CD^{pro}$.

Clinical studies using zinc supplementation are primarily limited to rhinovirus infection, and are often grouped with other "common cold" viruses such as influenza and coronaviruses. The majority of studies use zinc lozenges with various zinc formulations and concentrations, possibly explaining the large variability in results [extensively reviewed in (108) and (109)]. Importantly, the amount of ionic zinc present at the site of infection (oral and nasal mucosa) is highly correlated to the study outcome (51, 108), and is dependent on the zinc formulation. At a physiological pH and 37°C, zinc gluconate for example, releases high amounts of ionic zinc, whereas zinc aspartate releases none (108). Upon examining only the relevant studies where high doses of ionic zinc were used, a clear reduction in cold duration of 42% was calculated (109). Whether this was caused by viral inhibition, improved local immune response, or an amelioration of symptoms remains uncertain.

Other respiratory tract infections: influenza, coronavirus, and metapneumovirus.

Few studies have examined the antiviral effects of zinc on other respiratory viruses. In vitro replication of influenza (PR/8/34) is significantly inhibited by the addition of the zinc ionophore pyrrolidine dithiocarbamate (110), perhaps through inhibition of the RNA-dependent RNA polymerase (RdRp), as had been suggested 30 y earlier (111). In similar fashion, severe acute respiratory syndrome (SARS) coronavirus RdRp template binding and elongation was inhibited by zinc in Vero-E6 cells (60). Moreover, zinc salts were shown to inhibit respiratory syncytial virus, even while zinc was incubated with HEp-2 cells only before infection, and then removed (72). The authors suggest that this indicates an inhibitory mechanism similar to HSV by preventing viral membrane fusion; however, no measures were taken to assess changes in intracellular zinc content, nor inhibition of other aspects of the viral life cycle.

Flaviviridae: a focus on HCV.

Flaviviruses represent a number of insect-borne viruses including dengue and West Nile virus, as well as the hepatotrophic virus, HCV. The effect of zinc on insect-borne flaviviruses is scarce; however, in vitro studies by our group (34) and others (67) have demonstrated that zinc salts can reduce HCV replication (~50% at 100 μM $ZnSO_4$), perhaps by inhibiting the HCV RdRp, as shown in *E. coli* [half maximal inhibitory concentration (IC50) ~60 μM] (66). Although this is a potential mechanism, it has not been examined in eukaryotic cells in which zinc homeostasis is significantly different.

If left untreated, HCV becomes a chronic hepatic infection in around two-thirds of individuals (112), resulting in a significant reduction in plasma zinc (113). Consequently, zinc supplementation in HCV studies have focused on improved patient outcomes, particularly decreased liver inflammation, and enhanced response to antiviral treatment. Supplementation with 150 mg/d polaprezinc (a bioavailable zinc L-carnosine chelate) has been shown to reduce markers of hepatic inflammation alanine aminotransferase and aspartate aminotransferase alone (105), and in combination with the antiviral treatment IFN-α (106). Moreover, polaprezinc significantly improved the rate of viral clearance, particularly in patients with lower viral loads at baseline (102). The mechanisms underlying these observations remain uncertain; however, are likely a combination of direct antiviral effects and strengthening of the antiviral response. Zinc supplementation and the antiviral response is reviewed below.

Togaviridae.

Like flaviviruses, togaviruses primarily consist of arthropod-borne viruses such as Semliki Forest virus, Western equine encephalitis virus, and Chikungunya virus. Viral infection occurs by receptor-mediated endocytosis, followed by fusion of virus and endosomal membranes, and particle release into the cytoplasm (114). Using liposome (76), red blood cell (115), and BHK-21 (77) cell model systems, zinc has been shown to efficiently inhibit membrane fusion of Semliki Forest virus and sindbis viruses. Zinc ions interfere with membrane fusion by binding to a specific histidine residue revealed on the viral E1 protein at low endosomal pH (77). Unfortunately, the in vivo relevance of this model is unclear because of the high concentration of zinc (>1 mM) used. Notably, concentrated zinc is present in vesicular zincosomes that are thought to serve as intracellular zinc storage vesicles (116). Similar to the mechanism used by macrophages to inhibit intracellular *Mycobacterium spp*., zincosome fusion to viral endosomes may inhibit key aspects of the viral life cycle such as togavirus membrane fusion.

Retroviridae: HIV.

Retroviruses are named after their ability to transcribe RNA into DNA using their unique reverse transcriptase (RT), consequently allowing integration of retroviral DNA into the host genome. The integrated provirus can then establish a latent infection for the life of the host and is a major barrier to virus cure strategies, particularly for HIV-1 (117). Similar to viral RdRps, zinc has also been identified as

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an inhibitor of retrovirus RTs (118, 119). Fenstermacher and DeStefano demonstrated in 2011 that Zn^{2+} cations can displace Mg^{2+} ions from HIV-1 RT, promoting the formation of an excessively stable, but incredibly slow and inefficient replication complex (70). Zinc was also shown to inhibit the HIV-1 protease in 1991 (68), and to inhibit viral transcription in 1999 (69), but has received little attention since, with the exception of molecular simulation experiments that identified the zinc-binding sites at the catalytic aspartate-25 residue (120). As stated above, HIV can also stimulate zinc influx into monocytes (33), which may appear contradictory based on its antiretroviral properties. Latently infected monocytes and macrophages, however, can act as viral reservoirs for HIV (121), and could therefore benefit from zinc-mediated inhibition of cell death. In fact, unlike the majority of $CD4^+$ T cells, low levels of replication in macrophages do not result in cell death (122), making them a viable reservoir, in addition to long-lived resting $CD4^+$ T cells, for viral recrudescence after cessation of antiretroviral treatment.

Zinc deficiency is common in HIV-infected individuals, where it is associated with inflammation (123), immunological failure (124), and death (125). A recent Cochrane Review examined the role of micronutrient supplementation in people living with HIV (126). Although a number of studies demonstrated beneficial effects of zinc supplementation, the majority were underpowered. The authors concluded that zinc supplementation probably increases blood zinc concentration (moderate certainty), and may increase $CD4^+$ counts (low certainty).

Unlike zinc supplements, prophylactic zinc gels have shown a substantial benefit to limit HIV infection in vivo. Complete protection against vaginal SHIV-RT (a simian HIV virus expressing the human RT) infection in macaques was obtained by pretreating animals with an antiviral gel containing 14 mM zinc acetate and 50 μM MIV-150, a reverse transcriptase inhibitor (127). When used alone, zinc acetate is a potent antiviral, providing 66% protection against SHIV-RT vaginal infection (56) and an EC50 <100 μM in peripheral blood mononuclear cells against a range of HIV strains (128). Importantly, zinc treatment did not affect viral titers in macaques that became infected, nor did it result in zinc resistant HIV mutants with conserved *pol* (RT) mutations. These data suggest that zinc may not interfere with the HIV RT, but instead inactivate free virus or prevent viral attachment/penetration as reported for HSV (48, 54).

Papillomaviridae.

HPVs are oncogenic viruses that infect basal epithelial cells, where they stimulate proliferation resulting in warts. Although cutaneous warts are usually self-limiting and harmless, mucosal strains of HPV (e.g. high risk HPV-16 and -18) are a primary cause of cervical cancers (129). HPV oncoproteins E6 and E7 in particular, are significant drivers of cell proliferation and resistance to cell death by stimulating the degradation of tumor suppressor p53 and pRb, respectively [reviewed in (130)]. Although nuclear zinc appears to enhance HPV replication (see ***Zinc homeostasis and viral infection***), exogenous zinc treatment (CIZAR, zinc chloride and citric acid anhydrous) can effectively inhibit production of viral oncogenic proteins E6 and E7 (71). The inhibition of E6 and E7 by zinc results in apoptosis of cervical carcinoma cells, as they regain the function of tumor suppressors p53 and pRb (71). The mechanism by which zinc downregulates E6 and E7 expression is unknown, but may be preceded by a zinc-driven blockade in another component of the viral life cycle.

It would appear that both topical and oral zinc supplementation strategies have proven tremendously effective for cutaneous and genital warts. Unfortunately, the vast majority of studies are either underpowered, lacking suitable controls, or single case studies. Nonetheless, a recent systematic review concluded that zinc supplementation was the most effective systemic treatment for cutaneous warts, when compared to other available options (131). It should be noted, however, that individuals with persistent viral warts are often zinc-deficient or have lower concentrations than their healthy counterparts (132). In fact, studies demonstrating the most significant responses to zinc treatment had engaged patients that were primarily zinc-deficient (>70 μg/dL) (92, 94). Nonetheless, 78% (94) and 100% (92) of patients showed clearance of lesions in response to oral zinc sulfate (10 mg/kg up to 600 mg/d) compared to 13% and 0% of the placebo group, respectively. Topical zinc formulations have also proved efficacious for treatment of viral warts. A small study using a 4-wk topical 10% zinc sulfate regimen for plane warts demonstrated an 86% response rate (6/7), compared to a 10% response rate (1/10) in the control group (93).

Recent work suggests that treatment of vaginal HPV infections with topical zinc formulations may benefit the millions of women that remain unvaccinated against HPV. A recent pilot study demonstrated that intravaginal infusion of 500 μM zinc citrate in women diagnosed with high-risk HPV resulted in a 64% clearance rate, compared to 15% in the control group (133). Additional studies in mice have demonstrated that MZC, a formulation containing MIV-50, zinc acetate, and carrageenan, efficiently inhibited vaginal and anorectal HPV-16 pseudoviral particle infection (134).

In summary, it is evident that zinc possesses antiviral properties against a number of viral species. Although mechanistic studies are lacking, zinc appears to inhibit viral protease and polymerase enzymatic processes, as well as physical processes such as virus attachment, infection, and uncoating (**Figure 1**). Unfortunately, these mechanisms have not been well scrutinized in clinical studies, where zinc may provide inexpensive and effective adjunct treatments for many viral infections.

The role of zinc in antiviral immune signaling

Ionic zinc possesses unique and distinct antiviral properties against a number of human viruses; however, the antiviral immune response led by IFNs is invariably required to clear infections. Zinc has been shown to contribute to a number of innate and adaptive immune signaling pathways that have

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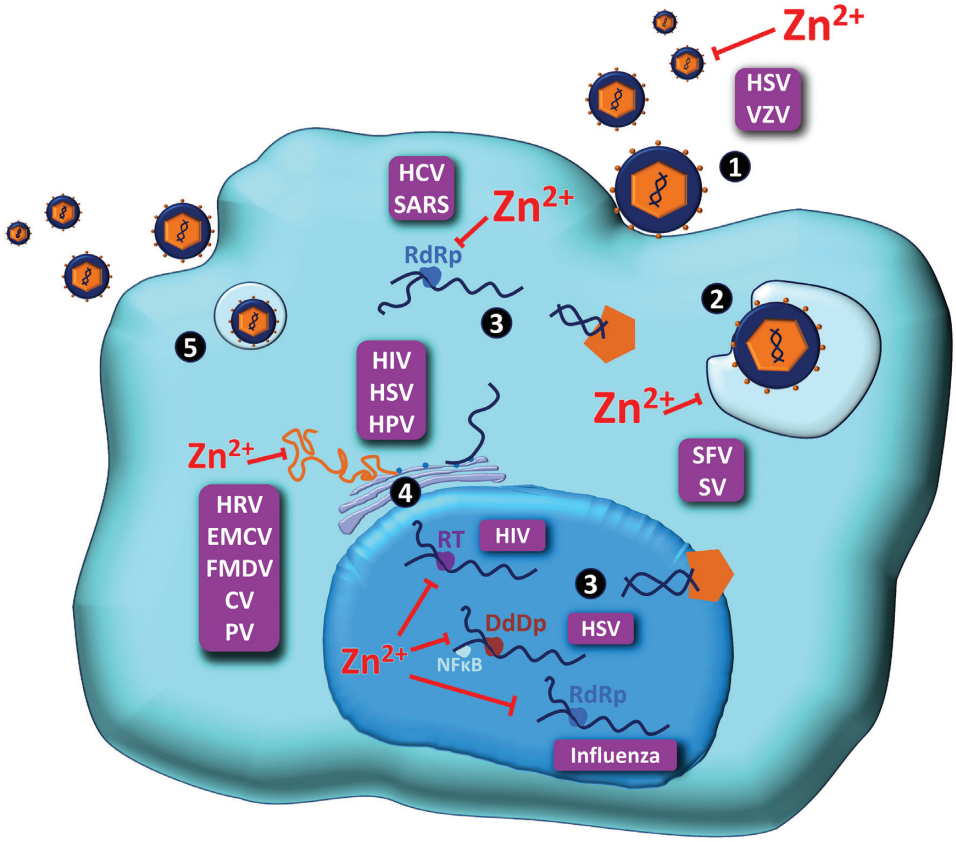


FIGURE 1 The diverse stages of viral replication cycles that are inhibited by zinc. In vitro studies have demonstrated a number of mechanisms by which zinc interferes with the viral replication cycle. These include free virus inactivation (1), inhibition of viral uncoating (2), viral genome transcription (3), and viral protein translation and polyprotein processing (4). No studies to date, however, have demonstrated zinc-mediated inhibition of virus assembly and/or particle release. CV, coronavirus; DdDp, DNA-dependent DNA polymerase; EMCV, encephalomyocarditis virus; FMDV, foot and mouth disease virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; HRV, human rhinovirus; HSV, herpes simplex virus; PV, polio virus; RdRp, RNA-dependent RNA polymerase; RT, reverse transcriptase; SARS, severe acute respiratory syndrome coronavirus; SFV, Semliki Forest virus; SV, sindbis virus; VZV, varicella-zoster virus; Zn, zinc.

been comprehensively reviewed recently (135). As such, this review will focus specifically on the role of zinc in the immune response to viruses.

Viral infections are recognized by a number of innate immune receptors termed pattern recognition receptors (PRRs). These include the cell surface and endosomal Toll-like receptors (TLRs), as well as a variety of cytosolic PRRs such as RIGI, MDA5, and IFI16 that primarily bind viral nucleic acids (136). Following ligand binding, PRRs share a number of downstream signaling intermediates, that ultimately activate both inflammatory (NF-κB, AP1) and innate immune (IRF1/3/7) transcription factors. These transcription factors cooperate to induce expression of IFNs, of which there are 3 types: type I (IFN-α and IFN-β), type II (IFN-γ), and type III (IFN-λs). Type I and III IFNs activate very similar antiviral signaling pathways; however, the type I IFN response is ubiquitous, whereas the type III IFN response is limited to a subset of immune cells, as well as epithelial cells of the liver, gastrointestinal, and pulmonary tracts (137). Although both IFN types bind unique receptors, they activate a common signaling cascade where STAT1 and STAT2 heterodimerize and bind IRF9, followed by translocation into the nucleus and subsequent binding of the IFN-sensitive response element that is present in hundreds of gene promoters. As stated previously, these ISGs possess numerous roles including immune cell chemotaxis and activation, as well as numerous antiviral mechanisms to inhibit viral replication within infected and neighboring cells.

Zinc and pathogen recognition.

Upon recognition of microbial antigens by TLRs, a rapid and transient influx of free zinc ions occurs. Interestingly, this has been demonstrated in response to viral stimuli, imiquimod, ssRNA40 (TLR7), and CpG (TLR9), but not polyI: C (TLR3) in the mouse macrophage RAW 264.7 cell line (138). In response to TLR7 activation, zinc was shown to reduce the production of type I IFNs and ISGs CD80

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and CD86. Based on results using other stimuli, the authors suggest that zinc can inhibit IRF3-, and perhaps IRF7-dependent IFNB production, by limiting activation and/or nuclear translocation (138). The role of the zinc influx in this context remains undefined, but may reflect a regulatory mechanism to prevent excessive IFN production.

Although no direct inhibition of IRF signaling by zinc has been demonstrated, zinc can modulate a number of factors upstream of IRF activation. For example, the IκB kinase (IKK) members IKKα and IKKβ are inhibited by zinc, albeit at high concentrations of ∼0.5 μM (139). IKKα has been shown to activate IRF7 in response to TLR7/9 stimulation (140), whereas IKKβ (141), IKKε and TANK-binding kinase-1 (TBK1) (142) can activate IRF3 following TLR3 stimuli. Zinc can also stimulate expression of the deubiquitinating enzyme A20 (43) to inhibit the pathogen response. A20 is a regulator of NF-κB- (143), TLR3- (144), and RIGI-mediated (145) IFN production, most likely by targeting PRR signaling components TIR-domain-containing adapter-inducing interferon-β (TRIF), TNF Receptor Associated Factor (TRAF) 2, and TRAF6. A20-deficient cells are hyper-responsive to viral infection, possess increased activation of NF-κB, IRF3, and IRF7, and improved viral clearance (146).

Zinc and the interferon response.

After pathogen recognition, NF-κB, AP1, and IRF3/7 bind IFN promoters to stimulate type I/III IFN production. Zinc plays a significant role in the response to IFNs by modulating secretion, cytokine potency, and receptor binding, as well as influencing signaling intermediates and pathway inhibitors. A recent study has demonstrated that intracellular zinc can reduce IFN secretion by destabilizing *sortilin* mRNA transcripts (147). Sortilin is an endosomal protein that facilitates secretion of cytokines such as IFN-γ and IL6 (148), and its depletion results in a significant reduction in secretion of IFN-α. Consequently, because sortilin ensures trafficking and secretion of numerous cytokines, it is possible that zinc also inhibits the secretion of other IFNs.

Structural studies have demonstrated that zinc ions can mediate dimerization of IFNA molecules (149). Nonetheless, apart from crystallization studies, dimers were difficult to generate despite using concentrated IFN (50 μM) and zinc (1 mM). It is therefore likely that the circulating active form of IFN-α is monomeric. A single study performed in 2001 showed that zinc can increase the antiviral activity of IFN-α 10-fold against rhinovirus challenge (150). Although this study drew radical conclusions, antiviral activity was based on cytopathic effect alone, and its results have not been reproduced since. Moreover, zinc was added before viral infection, which is known to interfere with rhinovirus polyprotein processing (73, 74), as reviewed above.

Unlike type I IFNs, a recent study by our group has shown that zinc can inhibit IFN-λ3 signaling, most likely by preventing receptor binding and subsequent signaling (28). Upon demonstrating in 2014 that metallothionein expression was *IFNL* genotype-dependent, and inversely associated with ISG expression in HCV (151), we showed that serum zinc was the driver of hepatic metallothionein expression. Although zinc had minimal effect on IFN-α signaling, it could almost ablate IFN-λ3 signaling at a concentration of 50 μM, resulting in a significant reduction in its antiviral activity (28). Interestingly, we found no inhibition of IFN-λ1 activity using 50 μM $ZnSO_4$, suggesting a highly specific interaction. The mechanism by which zinc interferes with the IFN:receptor interaction remains uncertain; however, we have ruled out an effect of zinc on IFN-λ3 disulfide bond formation.

Type I and III IFNs bind to unique receptor complexes composed of IFN-α receptors IFNAR1/IFNAR2 and IFN-λ receptors IFNLR1/IL10RB, respectively, but signal via almost identical pathways. Consequently, zinc may act to reinforce the shared IFN signaling cascade by inhibiting protein tyrosine phosphatase enzymatic activity (152). Following receptor engagement by IFNs, intracellular Janus protein tyrosine kinases Jak1 and Tyk2 become phosphorylated, which in turn phosphorylate STAT molecules to stimulate ISG expression. By dephosphorylating these key signaling molecules, a number of phosphatases have been shown to "put the brakes" on IFN signaling. Phosphatases tyrosine-protein phosphatase non-receptor type 6 (SHP1), type 11 (SHP2), and protein phosphatase 2A (PP2A) have all been shown to inhibit JAK-STAT phosphorylation (153–155), and are all inhibited by zinc ions, predominantly in the nanomolar range (156–158). Interestingly, PP2A can also inhibit the phosphorylation of IRF3, thus regulating antigen recognition by PRRs (159). Conversely, the tumor suppressor phosphatase and tensin homologue (PTEN) stimulates IRF3 activation by removing inhibitory phosphorylation at Ser97 (160), and is also inhibited by zinc at nanomolar concentrations (161). Zinc inhibits numerous pro- and antiviral phosphatases, with the net effect on virus recognition and response being undefined, which clearly requires further study.

To enable a highly controlled IFN response, negative regulators of IFN signaling are often ISGs. These include the suppressors of cytokine signaling (SOCS-1 and SOCS-3), which bind and inhibit JAK protein signaling, thus preventing signaling from numerous inflammatory (IL-6) and antiviral stimuli (162). Interestingly, zinc-driven activation of the MTF-1 transcription factor can induce expression of SOCS-3 in HepG2 cells (163). The zinc importer ZIP-14, which is responsible for zinc influx following inflammatory stimuli, was required for SOCS-3 expression, and may represent yet another zinc-mediated mechanism to limit the inflammatory response. Although the transporter responsible for hepatic zinc influx following IFN stimulation remains unknown, it is perceivable that ZIP-14 may drive zinc influx and subsequent SOCS-3 expression.

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Zinc deficiency caused by disease, age, and lifestyle factors: lessons from supplementation

Zinc status is primarily determined by dietary zinc intake; however, additional factors such as dietary composition, alcohol intake, and disease state can significantly reduce zinc uptake and storage, or increase zinc excretion (164). With respect to dietary composition, zinc supplementation as part of a meal can significantly reduce zinc absorption when compared to water-based solutions of zinc (164). Moreover, dietary phytate, a natural chelator of zinc ions that is present in corn, rice, and cereals, can severely restrict zinc absorption (165). Consequently, diets containing high phytate: zinc molar ratios, can result in zinc deficiency, even with adequate zinc intake. Unfortunately, rural diets in low-income nations are often zinc-poor and phytate-rich because of a dietary reliance on rice and vegetables.

Aged individuals are also significantly more susceptible to zinc deficiency, increasing their likelihood of acquiring life-threatening viral infections (166). Ex vivo, zinc supplementation has been shown to improve leukocyte IFN-α production (167) and to reduce mononuclear cell TNF production (168). Year-long supplementation with 45 mg elemental zinc/d in elderly subjects (aged 55–87 y), has also demonstrated a dramatic reduction in the incidence of infection as well as plasma oxidative stress markers (168).

Alcoholism can stimulate severe zinc deficiency developed via numerous sociological and physiological mechanisms, with factors including but not limited to *1*) increased urinary zinc excretion (169), *2*) reduced zinc intake (poor diet) (170), *3*) reduced zinc absorption (171), and *4*) and a reduction in hepatic zinc stores (172). Alcohol also stimulates microbial dysbiosis and gastrointestinal permeability (173), a phenotype that can increase the likelihood of viral infection in the gut (174). Importantly, dietary zinc supplementation can improve intestinal barrier dysfunction as a result of alcohol and microbial infection (175, 176).

As previously discussed, zinc deficiency is common among chronic infections such as HPV, HCV, and HIV (113, 123). Consequently, a number of studies have examined the effects of zinc supplementation on antiviral immunity, inflammation, and treatment response. As described above, zinc supplementation can improve HCV treatment response and liver inflammation caused by chronic infection. In addition, long-term zinc treatment over 7 y has been shown to reduce the risk of hepatocellular carcinoma progression in chronic HCV patients, as assessed by multivariate analysis, compared to controls ($P < 0.05$) (105). Zinc supplementation has also been assessed as an adjunct therapy to antiretroviral administration in patients with HIV. One study reported a 4-fold reduction in the rate of immune failure, as well as decreased diarrhea in patients treated with zinc compared to controls ($P < 0.05$ for both groups) (124). A more recent study revealed an increase in $CD4^+$ T cell count in patients treated with a combination of zinc and antiretroviral therapy, compared to patients on antiretroviral therapy alone ($P < 0.05$) (177). Taken together, these data indicate that zinc deficiency is associated with greater disease activity in the context of chronic viral infection. Oral zinc supplementation may act in a synergistic manner when co-administered with antiviral therapy and contribute to improved clinical outcomes.

Vaccination studies.

Zinc supplementation during vaccination strategies has provided an opportunity to examine the role of zinc in the humoral response to viruses. A particular focus has been applied to the effect of zinc supplementation on rotavirus vaccination because of the high rate of mortality associated with childhood diarrhea in developing countries. Unfortunately, although zinc deficiency is associated with increased risk of rotavirus gastroenteritis (178), it does not greatly increase the development of humoral immunity followed by vaccination (rotarix), as defined by seroconversion rate (179). Nonetheless, a pooled analysis of randomized trials performed in 2000, demonstrated that zinc supplementation shortens the length of diarrheal episodes and reduced the rate of treatment failure or death by 42% in zinc-deficient children (180).

Comparable studies of supplementation with zinc before vaccination have produced similar disappointing results. Zinc supplementation did not improve seroconversion following administration of the oral poliovirus vaccine in infants (181), nor did it improve the immunological response to HBV (182) or influenza vaccination (183) in the elderly. Although there remains little evidence that zinc improves viral vaccination responses, a small number of studies suggest that zinc may improve antibody titers and antibacterial responses to pneumococcus (184) and cholera infections (185).

Conclusions and Future Perspectives

The tight regulation of zinc homeostasis both systemically and intracellularly indicates that zinc plays an essential role in human health. Although zinc is a component of ~10% of the human proteome, zinc in different forms (free compared with protein-bound) can stimulate a variety of signaling events, including the antiviral response. In vitro studies suggest that free zinc may possess potent antiviral effects, and are supported by trials of creams, lozenges, and supplements with high free zinc content. Moreover, zinc-binding proteins such as the metallothioneins may possess antiviral roles, although their specific function remains uncertain. Nonetheless, zinc treatment applied at a therapeutic dose and in the right form has the potential to drastically improve the clearance of both chronic and acute viral infections, as well as their accompanying pathologies and symptoms. Consequently, the role of zinc as an antiviral can be separated into 2 categories: *1*) zinc supplementation implemented to improve the antiviral response and systemic immunity in patients with zinc deficiency, and *2*) zinc treatment performed to specifically inhibit viral replication or infection-related symptoms (75, 78–82, 83, 85–91, 95–101, 103, 104).

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