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ORIGINAL ARTICLE

Adenovirus 5 produces obesity and adverse metabolic, morphological, and functional changes in the long term in animals fed a balanced diet or a high-fat diet: a study on hamsters

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Abstract

Adenovirus 5 (Ad-5) infection is a common cause of acute respiratory infections and the main vector used in gene therapy. There are few studies on the relationship of Ad-5 to obesity. In the present study, we evaluated the chronic effects of Ad-5 infection on golden (Syrian) hamsters fed either a balanced diet (BD) or a high-fat diet (HFD). After a single inoculation with Ad-5 (1×10^7 pfu), the body weight of the animals was measured weekly. Medium-term (22 weeks) serum biochemical analyses and long-term (44 weeks) liver morphology, adiposity, and locomotive functionality (movement velocity) assessments were carried out. In the animals fed the BD, adenovirus infection produced hyperglycemia and hyperlipidemia. In the long term, it produced a 57% increase in epididymal pad fat and a 30% body weight gain compared with uninoculated animals. In addition, morphological changes related to non-alcoholic fatty liver disease (NAFLD) were observed. The animals fed the HFD had similar but more severe changes. In addition, the hamsters presented an obesity paradox: at the end of the study, the animals that had the most morphological and functional changes (significantly reduced movement velocity) had the lowest body weight. Despite the fact that an HFD appears to be a more harmful factor in the long term than adenovirus infection alone, infection could increase the severity of harmful effects in individuals with an HFD. Epidemiological studies are needed to evaluate the effect of adenovirus as a precursor of chronic liver and cardiovascular diseases, including the chronic effects of gene therapy.

Introduction

Obesity is a chronic disease characterized by excessive storage of adipose tissue in the organism. Its multifactorial etiology includes genetic, social, metabolic, endocrine, and neurological factors [1]. An infectious origin of obesity has been suggested, due to its rapid dissemination pattern [2]. The concept

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of virus-induced obesity is not new. Recent studies have suggested that adenovirus types 31, 36, and 37 can infect adipocytes and alter expression patterns, causing triglyceride accumulation, pre-adipocyte differentiation into mature adipocytes [3, 4], and increased glucose entry into cells [5, 6].

Most studies relating adenoviruses to obesity have been carried out with Ad-36, and there is no longer any doubt that it is adipogenic [3]. However, very little research has been conducted on type 9 or type 5 adenoviruses. So et al. reported that mice inoculated with adenovirus type 5 (Ad-5), whose food intake was not different from that of controls, had significant weight gain [7]. An epidemiological study associated Ad-5 seropositivity with a greater prevalence of obesity in children [8].

Epidemiological studies have demonstrated that Ad-5 is a very common virus in humans. Five to ten percent of all febrile illnesses in infants and young children are attributable to adenovirus infections, typically involving the respiratory tract and commonly caused by type 5 [9]. Studies carried out on different adult populations have shown a prevalence of anti-Ad-5 serum antibodies of 57% in the United States [10], 74% in the Netherlands [11], 77% in China [12, 13], 79% in South Africa, and 84% in Gambia [14]. Therefore, it is important to determine the long-term changes that Ad-5 infection can produce with respect to adipogenesis, metabolism, organ morphology, and the general health status of the individual.

The majority of *in vivo* trials on the effects of adenoviral infection have been performed on rats or mice. However, they are not the ideal rodent models for studying adenovirus infection, given that there can be no viral lytic cycle in their cells. Syrian (golden) hamsters (*Mesocricetus auratus*) have been shown to be more appropriate rodents for studying adenovirus infection because the virus can replicate in the liver, lungs, and most other organs [15–18]. The production of adenovirus infections in Syrian hamsters has been demonstrated through intravenous or intranasal inoculation [18], as well as via intraperitoneal inoculation [19, 20]. Syrian hamsters are also suitable models for studying obesity and dyslipidemia [12, 21, 22].

The present study was designed to evaluate the adipogenic effect and long-term biochemical, morphological, and functional disorders caused by Ad-5 infection in Syrian (golden) hamsters fed either a balanced diet or a high-fat diet.

Materials and methods

Cells and viruses

Human embryonic kidney (HEK)-293T cells were used (Thermo Fisher Scientific, Inc., Waltham, MA, USA). They were maintained in DMEM containing 10% fetal bovine serum (FBS) and supplemented with L-glutamine, penicillin, and streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO_2 in a humidified environment. Wild-type adenovirus type 5 (Ad5) was used. Viruses were grown on HEK-293 cells and purified using a ViraKit AdenoMini-24 kit (Virapur LLC, San Diego CA, USA) according to the manufacturer's instructions [23]. The viruses were titrated on HEK-293 cells by tissue culture infectivity dose 50 ($TCID_{50}$) as described previously in the Vector System Application Manual, version 1.4 (Virapur LLC, San Diego CA, USA).

Animals and study design

Female rodents, including hamsters, produce hormones that promote an increase in fat storage and body weight gain [24, 25]. To control the influence of that natural hormonal adipogenic factor on the present experiment, only male animals were selected. In addition, the hepatic lipoprotein metabolism of the male hamster has been found to closely resemble that of humans [26, 27]. The present study was therefore carried out using 40 male Syrian (golden) hamsters (HsdHan™:AURA; Envigo, Huntingdon, UK) (6-8 weeks old). They were handled under sterile conditions and kept in cages with filters to avoid contact with microorganisms. Conditions were controlled (12 h:12 h light/dark cycle, 23°C temperature with 50% humidity), and water was freely available. National and international norms for laboratory animal care were followed. Before the study was begun, the hamsters were acclimated to the animal laboratory environment for 15 days. Twenty animals were fed a high-fat Western diet (HFD) that contained 21.2% fat and 17.3% protein (TD.02028 Atherogenic Rodent Diet, Envigo, Huntingdon, UK), and 20 were fed a balanced rodent diet (BD) that contained 6.2% fat and 18.6% protein (2018S Tekland Global 18% Protein Rodent Diet, Envigo, Huntingdon, UK). Half of the animals from each dietary group were inoculated with a single application of adenovirus type 5, forming four groups for the experiment: two groups fed the BD (one group infected with Ad-5 and the other not infected with Ad-5, as the control) and two groups fed the HFD (one group infected with Ad-5 and the other not infected with Ad-5, as the control). The placement of animals in the different groups was random.

For adenovirus type 5 inoculation (infected groups), 1×10^7 infectious Ad-5 viral particles (pfu) were dissolved in 100 µl of DMEM and administered intraperitoneally. Physiological saline solution was administered to the control groups. The experimental animals were monitored through direct observation, at least once during the first 30 minutes, periodically during the first 24 hours, and then at least once a day to qualitatively detect any adverse clinical signs,

following the guidelines of the Organisation for Economic Co-operation and Development [28, 29]. The adverse clinical signs detected in the acute infection phase are described in the Results and Discussion sections. One venous blood sample was drawn from the animals at post-inoculation day 3 and week 6. DNA was isolated from those samples by the standard method (proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation) [30]. The presence of adenovirus was determined from that DNA through real-time PCR, using a TaqMan assay, with oligonucleotides and a probe designed for the adenoviral E1A terminal region as described previously [31]. Animal body weight was determined every week for 44 weeks. At week 22 of that period, the medium-term effect was assessed through measurements of body weight, serum cholesterol, high-density lipoprotein (HDL), albumin, globulin, and glucose (with 4 h of fasting). Non-HDL cholesterol was computed as the difference between total cholesterol and HDL cholesterol [22]. Those biochemical analyses were performed in triplicate using an automatic biochemical analyzer (Cobas c111, Roche ®, Mexico). The long-term effect (follow-up at week 44) was analyzed through body weight measurement and locomotor activity evaluation. The animals were then euthanized by decapitation, their epididymal fat pads and livers were weighed, and histopathologic liver analysis was carried out. The epididymal fat pad is very well delineated in hamsters and has been used as a parameter for determining alterations in fat accumulation in animals (the adipogenic effect, when an increase in fat is found) [32].

The hamsters were observed for 44 weeks. Taking into account the age of the animals upon their entrance into the study, the final analyses were carried out on animals that were 52-54 weeks old. Three factors were considered for selecting that period of observation: 1) under normal laboratory conditions, animals gain their maximum weight at 20 to 30 weeks [21, 33–35]; 2) changes in animal behavior related to the aging process begin at 50 weeks of life [36]; and 3) animal death has been reported to start a little after week 50 and can begin even sooner if the animals are exposed to damaging factors, such as cigarette smoke [37]. Therefore, the length of time the animals were followed in the present study made it possible to observe them from the stages of youth to old age without entering the accelerated mortality stage.

Locomotor activity evaluation

At week 44, the locomotor activity of the hamsters was evaluated through an open field activity monitoring system, which is a useful tool for assessing locomotive impairment in animal models utilizing small rodents, including hamsters [38–42]. For that trial, a cube with 55 × 55 cm acrylic walls was used as the open field. Each hamster was placed in the center of the cube and allowed free exploration for 5 min. When that time was up, the animal was removed from the cube and returned to its box. The session was videotaped and evaluated using EthoVision XT7 video tracking software (Noldus Information Technology, Inc. Leesburg, VA, USA), thus determining the motional velocity of the hamster (cm/s). After each trial, the instrument was cleaned to return to standard conditions [43]. A significant change in velocity, compared with a control, was considered an alteration in the locomotor activity of the experimental animal [44].

Histopathologic liver analysis

Liver tissues were fixed in solutions of 10% formaldehyde for 5 days at 24°C. Two cross-sectional slices were made from the right lobe (the central region and external third region), and one slice was made from the left lobe. The specimens were dehydrated in ethanol, embedded in paraffin wax, sectioned (5-µm thick), and stained with hematoxylin and eosin at 24°C for 5 and 2 min, respectively. Glycogen storage was observed through periodic acid-Schiff staining (PAS). The tissue slices were placed in the Schiff reagent for 15 min, rinsed in water for 10 min, and counterstained with Mayer's hematoxylin for 1 min. The slices were evaluated via images captured with an Axiocam MRC-5 model digital camera (Zeiss GmbH, Jena, Germany) attached to an AxioPlan 2 M model bright-field optical microscope (Zeiss GmbH, Jena, Germany) with a motorized stage and A-plan x20 objective. Images (200X) of the entire surfaces of the samples were assembled using the MosaiX and Autofocus modules. All images were captured under the same conditions of light and exposure. The same blinded pathologist performed the analyses using AxioVision software version 4.0 (Zeiss GmbH, Jena, Germany) [45, 46].

Binucleated cells were identified in the images of the entire surfaces of the liver samples (stained with hematoxylin/eosin), and their percentage in relation to the total number of cells was calculated. The number of cells per square millimeter was also determined. The areas with degenerative data were manually marked in the interactive mode to calculate the percentage of the area with liver degeneration. The percentage of the area with glycogen was calculated in a similar manner, but also included the measurement of the areas stained with PAS.

Steatosis was considered in relation to the percentage of liver tissue with fat accumulation [47]. According to previously described methodology and classification, inflammation was evaluated through the functioning histologic zones, depending on the oxygen supply: zone 1 encompassed the portal tracts, where the oxygenated blood from the hepatic arteries entered; zone 3 was located around the central veins, where oxygenation was poor; and zone 2 was located between zones 1 and 3. There were four categories

in relation to the percentage of tissue with inflammatory infiltrate: none, mild, moderate, and severe (0%, up to 33%, 33-66%, and more than 66%, respectively) [48]. The histopathologic analyses were performed on the digital images of the entire surface of each liver sample (right and left lobules) at a magnification of X200, with 3000 to 6000 individual fields merged into one panoramic image. The pathologic changes were quantified per area and compared accordingly.

Statistical analysis

Mean and standard deviation were used for the descriptive statistics. For the inferential statistics, normal data distribution was first determined through the Kolmogorov-Smirnov test, and the equality of variances was confirmed using Levene's test. One-way ANOVA was used to analyze differences between groups. A further in-depth post-hoc analysis of the variation was carried out using Student's *t*-test, which enabled the differences between each of the groups to be determined. Statistical tests were performed using IBM SPSS version 20 software (IBM SPSS, Chicago, Illinois, USA). Statistical significance was set at $P < 0.05$.

Bioethical issues

The trials complied with the national and international legal and ethical requirements applicable to pre-clinical research. The experimental protocols were approved by the Research Ethics Committee of the School of Medicine of the *Universidad de Colima*, Mexico (Protocol Number: UCOL14-016). The animals were handled according to institutional guidelines, the Mexican official norm regulating laboratory animal use (NOM-062-ZOO-1999), and the Guide for the Care and Use of Laboratory Animals issued by the National Academy of Sciences of the United States of America (2011). All animals were euthanized (by decapitation) according to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition.

Results

Ad-5 infection in hamsters

The presence of adenovirus particles in blood was confirmed by real-time PCR on the third day after inoculation with Ad-5. The animals inoculated with the virus were positive for Ad-5, and those of the control groups (not inoculated with Ad-5) were negative. At post-inoculation week 6, all of the animals (infected and controls) were negative for the presence of adenoviral DNA in blood samples, determined through real-time PCR, demonstrating that the infection was self-limited and acute. Adynamia was observed in the infected group the day after Ad-5 inoculation. Diaphoresis, chills, and reduced food intake were observed on days 2 and 3. Signs reverted on days 4 and 5, except adynamia. Adynamia disappeared between days 6 and 7, and the animals regained a "normal" aspect (the same as that of the non-infected hamsters). There were no alterations in aspect or behavior in the group inoculated with saline solution.

Body weight gain: short-term, medium-term, and long-term effects

As shown in Figure 1A, no differences in mean weight were observed in the groups fed the balanced diet from the beginning of the study. However, beginning at week 8 and continuing to the end of the study, the body weight of the infected animals was significantly higher than the body weight of the control-group animals. At the medium-term (weeks 8-22), the body weight of the Ad-5-infected animals was on average 4 to 6% higher than that of the control-group animals ($P < 0.05$). The long-term effect (at week 44) was striking, with the body weight of the Ad-5-infected animals being 30% higher than that of the non-infected animals ($P < 0.0001$; see Fig. 1A and the Electronic Supplementary Material for further details).

There was rapid weight gain in the animals fed the high-fat diet during the first weeks. From week 3 to week 16, the body weight of the uninfected animals fed the high-fat diet was significantly higher than that of the animals fed the balanced diet ($P < 0.05$). From week 22, all of the groups, except the adenovirus-infected group fed a balanced diet, began to gradually lose weight. This weight loss was more accelerated in the animals fed the high-fat diet. Thus, from week 22, the body weight of the animals fed the high-fat diet was significantly lower than that of the animals fed the balanced diet ($P < 0.05$; see Electronic Supplementary Material for further details). No significant changes were observed between the infected and uninfected animals fed the high-fat diet, except at weeks 22 ($P = 0.003$) and 36 ($P = 0.04$), in which the infected animals had an approximately 10% higher body weight than the animals in the uninfected group (see Fig. 1B and Electronic Supplementary Material). No significant differences in body weight were observed in the long term at 44 weeks between the infected and uninfected animals fed the high-fat diet (see Fig. 1B and Electronic Supplementary Material for further details).

Serum biochemical parameter alterations: medium-term effects

In the medium term at week 22, metabolic changes caused by Ad-5 infection were observed. Acute Ad-5 infection in the animals fed the balanced diet caused significant elevation in globulin, glucose, total cholesterol, and non-HDL

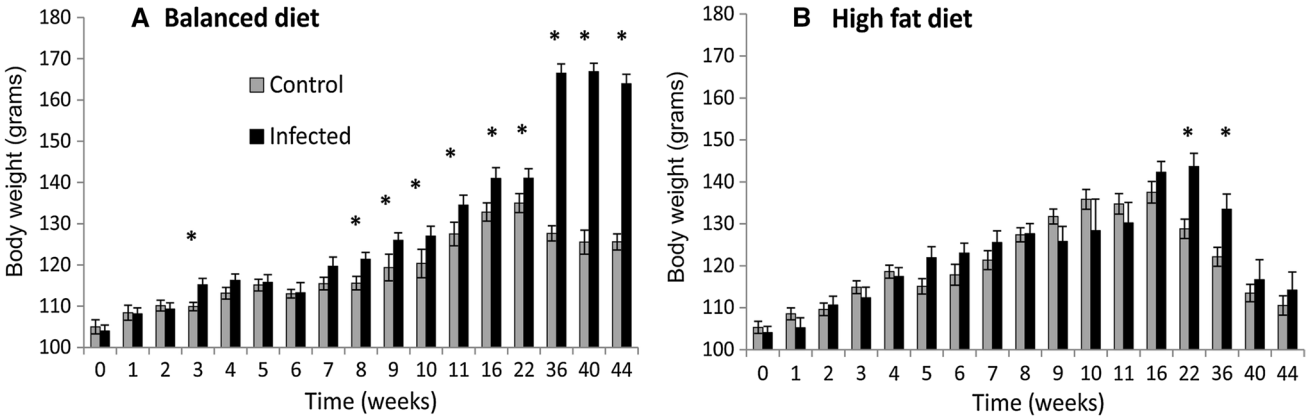

Fig. 1 Body weight of the animals fed the balanced diet (A) and the high-fat diet (B). The Ad-5-infected animals fed the balanced diet had significant and continuous weight gain from week 8, with a 30% increase at the end of the study, with respect to the controls. The Ad-5-infected animals fed the high-fat diet had significant weight gain only at weeks 22 and 36. Reduced body weight was observed in most of the animals, regardless of group. A similar event has been reported within the life cycle of Syrian hamsters (see Discussion). Mean and standard deviation are plotted. *, $P < 0.05$

cholesterol levels, together with a reduction in HDL cholesterol levels, compared with the uninfected control group animals (Table 1). Acute Ad-5 infection in the animals fed the high-fat diet caused no relevant changes in the parameters studied, except elevated total cholesterol levels. It should be pointed out that the high-fat diet itself caused significant changes in all of the study parameters when compared with the animals fed the balanced diet (Table 1).

Long-term effects: epididymal fat pad weight gain

The epididymal fat pads of male rodents are frequently sampled because they are often used as a proxy of visceral fat [49] and adipogenesis [50]. The epididymal fat pad bulk mass at week 44 is shown in Fig. 2A. In the animals fed the balanced diet, epididymal fat pad weight was 57% higher in the animals infected with Ad-5 than in the uninfected controls ($P = 0.0002$). No significant differences were observed between the infected and uninfected animals fed the high-fat diet ($P = 0.20$). Comparisons between the animals fed a balanced diet and those fed a high-fat diet are shown in Electronic Supplementary Material.

Long-term effects: locomotion function

The movement velocity of the animals in an open field was determined. As shown in Fig. 2, no significant differences were observed between the infected and uninfected animals that were fed the balanced diet (8.0 ± 2.3 vs. 9.6 ± 2.9 cm/s, respectively). However, in the animals fed the high-fat diet, those infected with Ad-5 had significantly lower movement velocity than the uninfected animals (6.1 ± 0.6 vs. 9.4 ± 2.4 cm/s, $P < 0.05$) (Fig. 2C).

Long-term effects: liver morphology

Figure 2B shows the hamster liver weight. The livers of the infected animals fed the balanced diet weighed 22% more than those of the uninfected animals of the control group ($P = 0.001$). No significant differences in liver weight were observed between the infected and uninfected animals in the high-fat diet groups ($P = 0.56$). It is important to mention that the livers of the animals fed the high-fat diet weighed significantly more than the livers of the animals fed the balanced diet (uninfected control groups: 7.8 ± 1.0 vs. 5.4 ± 0.4 grams, $P = 0.0002$; infected groups: 8.2 + 0.7 vs. 6.7 ± 0.8 grams, $P = 0.001$, in the animals fed the high-fat diet vs. those fed a balanced diet, respectively). In the comparison of liver weights of the animals exposed to a single risk factor (Ad-5 infection or high-fat diet), the livers of the uninfected animals fed a high-fat diet weighed more than the livers of the Ad-5-infected animals fed the balanced diet (7.8 ± 1.0 vs. 6.7 ± 0.8 grams, respectively; $P = 0.004$). Those results showed greater hepatomegaly caused by the high-fat diet than by Ad-5 infection, making it clear that diet was more relevant than infection in relation to long-term liver alterations.

No steatosis was present in the liver tissues analyzed by microscopy. All of the livers of both the infected and uninfected animals fed the high-fat diet had mild inflammation, whereas the livers of the animals fed the balanced diet had no signs of inflammation. Table 2 shows that there were no significant differences in the number of hepatocytes per square millimeter between the Ad-5-infected animals and the uninfected controls. Thus, it can be assumed that the increased liver weight in the infected animals was due to an increase in the total number of cells (hyperplasia) and

Table 1 Comparison of different biochemical parameters according to type of diet and Ad-5 exposure at week 22 of follow-up (medium-term effect)

Parameters per group	Diet		*P***
	Standard	High-fat	
Glucose			
Control	79.6 ± 20.8	92.5 ± 19.4	0.040
Infected	107 ± 17.1	101.7 ± 21.5	0.250
*P****	0.004	0.35	
Cholesterol			
Control	91 ± 3.1	226.3 ± 61.7	<0.001
Infected	90.5 ± 1.5	273.5 ± 47.5	<0.001
*P****	0.640	0.04	
HDL-C			
Control	32 ± 11.9	112.1 ± 51.3	<0.001
Infected	18.7 ± 10.6	148.8 ± 76.3	<0.001
*P****	0.009	0.140	
nHDL-C			
Control	59.0 ± 10.2	114.0 ± 53.7	<0.001
Infected	71.8 ± 8.8	124.0 ± 53.5	0.003
*P****	0.005	0.310	
Albumin*			
Control	2.52 ± 0.23	1.95 ± 0.31	0.001
Infected	2.65 ± 0.19	2.10 ± 0.32	<0.001
*P****	0.100	0.210	
Globulin*			
Control	3.58 ± 0.41	4.90 ± 0.36	<0.001
Infected	3.89 ± 0.30	4.60 ± 0.43	<0.001
*P****	0.024	0.101	

The values are expressed as mean ± SD (mg/dL or *g/dL). HDL, high-density lipoprotein cholesterol; nHDL-C, non-HDL cholesterol. Statistical analysis was carried out using Student's *t*-test

**Comparison of balanced diet vs. high-fat diet groups

***Comparison of uninfected vs. infected groups

not to an increase in cell size. The Ad-5-infected hamsters fed the balanced diet had an increase in the percentage of binucleated cells and a reduced glycogen area relative to the uninfected hamsters (Fig. 3). In the animals fed the high-fat diet, those infected with Ad-5 had a reduced glycogen area and an increased percentage of the area with liver degeneration compared with the uninfected animals (Table 2 and Fig. 3). These data show that Ad-5 infection caused chronic liver changes when compared with the animals that were not infected, regardless of diet. Nevertheless, the Ad-5-infected animals fed the high-fat diet had the most substantial changes in microscopic liver morphology.

Compared with the animals fed the balanced diet, the animals fed the high-fat diet had an increase in the number of hepatocytes per square millimeter and in the percentage of binucleated cells, as well as a reduction in the area occupied by glycogen. These changes occurred in both the infected and uninfected animals. In a comparison of hyperplasia and the increase in binucleated cells in the livers of the animals exposed to only one risk factor (Ad-5 infection or high-fat diet), we found that the livers of the uninfected animals fed the high-fat diet had higher values in both parameters than the Ad-5-infected animals fed a balanced diet. Greater hyperplasia and hepatocyte binucleation were caused by the high-fat diet rather than by Ad-5 infection, suggesting that diet is more relevant than infection in relation to long-term microscopic liver alterations (Table 2).

Discussion

It was possible to determine that a single and transitory infection with adenovirus type 5 was capable of promoting significant weight gain in hamsters fed a balanced diet. In the long term, the animals with Ad-5 infection had a

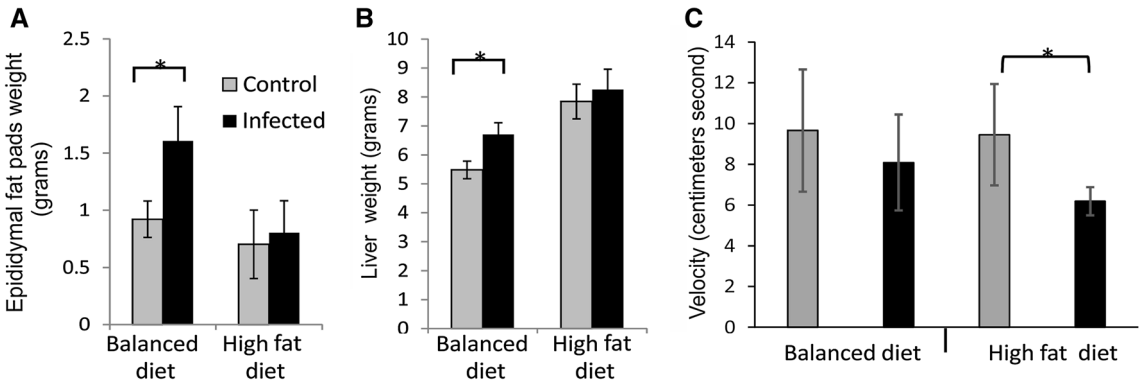

Fig. 2 Epididymal fat pad weight (A), liver weight (B), and movement velocity of the animals (C) in the long term (week 44). There was a significant increase in the weight of the epididymal fat pads and livers of the Ad-5-infected animals fed the balanced diet relative to the uninfected controls. There were no differences in the weights of these organs in the animals fed the high-fat diet. Regarding the movement velocity (cm/s) of the animals (C), only the Ad-5-infected animals fed the high-fat diet had significantly lower motional velocity when compared with the other groups. Mean and standard deviation are plotted. *, $P < 0.05$

Table 2 Comparison of the different histological parameters according to type of diet and Ad-5 exposure at week 44 of follow-up (long-term effect)

Parameters per group	Diet		
	Standard	High-fat	*P**
Hepatocytes/mm^2			
Control	2.36 ± 0.30	3.11 ± 0.40	<0.001
Infected	2.49 ± 0.29	3.31.7 ± 0.31	<0.001
*P***	0.320	0.190	
% binucleated cells			
Control	8.6 ± 2.2	15.1.3 ± 4.2	<0.001
Infected	11.1 ± 2.5	12.1 ± 4.2	0.260
*P***	0.010	0.150	
% of nuclear area			
Control	16.0 ± 2.5	30.5 ± 1.6	0.003
Infected	30.8 ± 2.1	30.2 ± 2.9	0.330
*P***	0.006	0.420	
% of area with glycogen			
Control	20.1 ± 8.6	3.9 ± 2.7	<0.001
Infected	10.6 ± 3.7	0.9 ± 0.9	<0.001
*P***	0.030	0.040	
% of area with degeneration			
Control	16.0 ± 5.6	19.9 ± 6.3	0.100
Infected	16.5 ± 6.6	40.2 ± 12.5	<0.001
*P***	0.420	0.002	

Statistical analysis was carried out using Student's *t*-test

*Comparison of balanced diet vs. high-fat diet groups

**Comparison of uninfected vs. infected groups

30% higher body weight and a 57% higher accumulation of epididymal pad fat. In addition, Ad-5 exposure caused metabolic changes in the medium term (week 22), with clear hyperglycemia and hyperlipidemia. In the long term (week 44), hyperplasia, increased binucleation, and an increased nuclear area at the hepatic level were observed in the animals with Ad-5 infection fed a balanced diet, as well as a decreased percentage of liver tissue with glycogen. Binucleation (polyploidization) is one of the most dramatic changes that can occur in the genome [51–53]. In the liver, physiological polyploidization events occur both during liver development and throughout adult life [51–53]. However, pathological polyploidization is known to take place in the early stages of nonalcoholic fatty liver disease (NAFLD), which is promoted by an increase in oxidative stress [51–53]. Liver hyperplasia has also been confirmed in the earliest stage of NAFLD in *ob/ob* mice, identifying this characteristic as an obesity-related metabolic abnormality [54]. In addition, reduced hepatic glycogen is a characteristic that has previously been associated with chronic liver damage [55].

Obesity, hyperlipidemia, hyperglycemia, and chronic liver damage (NAFLD) are systemic disease mediators that are associated with increased liver-related complications and liver-related mortality, as well as an increased risk for developing type 2 diabetes, cardiovascular disease (CVD), chronic kidney disease, and certain malignancies, including primary liver cancer and colorectal cancer [56–59]. Thus, the results of the present study are relevant, because in addition to showing the obesogenic effect of Ad-5 infection in

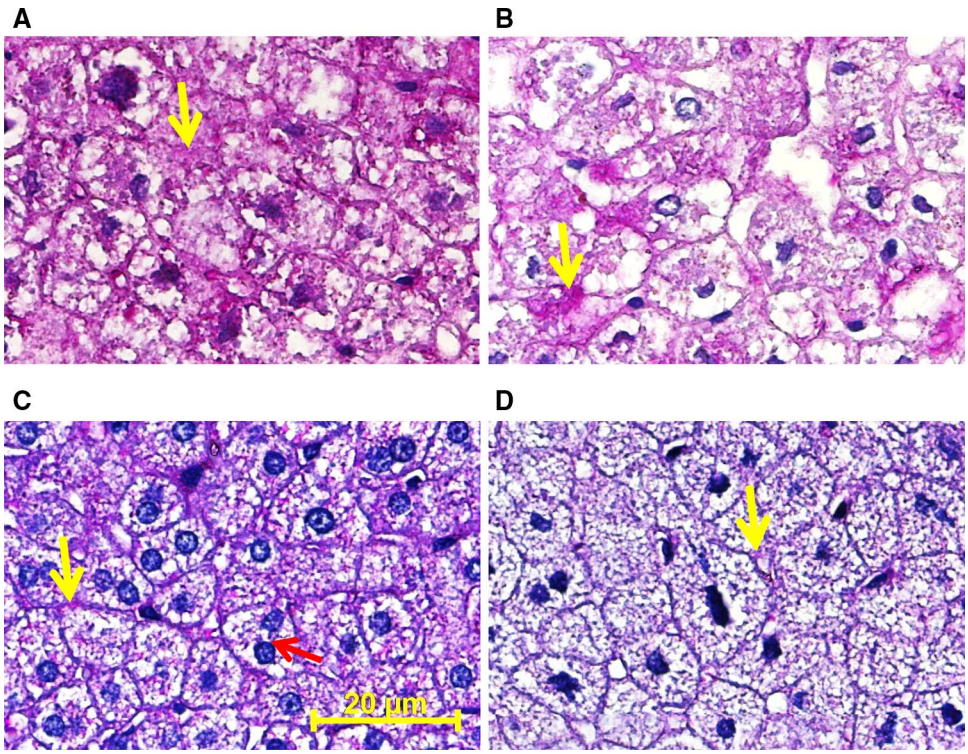

Fig. 3 Images showing the liver morphology of uninfected animals fed a balanced diet (A), Ad-5-infected animals fed a balanced diet (B), uninfected animals fed a high-fat diet (C), and Ad-5-infected animals fed a high-fat diet (D). Images show periodic acid-Schiff staining (PAS) x200. The images mainly show the changes in the glycogen deposits, which are bright purple (#a00498 Color Hex) and indicated by yellow arrows. The deposits are abundant in panel A, gradually reduced in panels B and C, and extremely scarce in panel D. The red arrow indicates a binucleated cell (only for illustrative, not comparative, purposes). The results of the analysis of those parameters are shown in Table 2. The lower glycogen content is apparent in the infected animals vs. the uninfected animals

the long term, they also confirmed the induction of metabolic and hepatic morphology alterations that are associated with increased morbidity and mortality.

However, human obesity and its associated disorders have a multifactorial etiology. Their complexity cannot be fully explained by a single factor, such as Ad-5 infection alone, and therefore we also analyzed the effect of a high-fat diet in the present study. It was obvious that a high-fat diet, on its own, caused rapid weight gain in the medium term, with hyperglycemia and hyperlipidemia, when compared with a balanced diet. It also produced pathologic changes in liver morphology in the long term. In animals that were fed a high-fat diet, Ad-5 infection caused significant weight gain in the medium term (week 22) and resulted in an increase in the already higher total cholesterol levels, when compared with uninfected control animals that were fed a high-fat diet. In the long term (week 44), liver morphology, already affected by the high-fat diet, became more deteriorated in the animals exposed to Ad-5, and there was a considerable reduction in the amount of glycogen and an increase in the degenerated areas. Liver degeneration also reflects damage previously associated with NAFLD [60]. The significant reduction of serum albumin in the animals fed the high-fat diet, regardless of Ad-5 infection (Table 1), coincided with the greater liver damage found in the HFD groups at the end of the study. Reduced albumin levels have previously been related to obesity [61, 62] and to NAFLD [63, 64] and are an important morbidity indicator in chronic diseases [65, 66]. In general, our results revealed that both a high-fat diet and adenovirus type 5 infection, each on their own, caused adverse health events in the long term. Health was damaged more in animals with only a high-fat diet than in animals that had a balanced diet plus Ad-5 infection, but the most severe alterations were found in the group with the combination of a high-fat diet and Ad-5 infection.

Hamster motional velocity was significantly reduced only in the animals with both Ad-5 infection and a high-fat diet. That is a relevant result because it is the first time adenovirus infection has been shown to cause functional alterations in animals in the long term, even though the alterations were only observed when the infection was combined with HFD. The authors of a recent study on humans reported that the presence of a cardiovascular risk factor burden, especially when combined with other factors, may increase the risk of walking speed limitation in older adults under 78 years of age, independent of cognitive function [67]. In the present study, the significant reduction in the movement velocity of the animals infected with Ad-5 and fed a high-fat diet could represent a greater cardiovascular compromise than in the animals of the other study groups.

The body weight loss observed in the animals at the end of the study (Fig. 1) could be associated with the life cycle of Syrian hamsters, given that they reach their maximum body weight at 20 to 30 weeks of age, after which they begin to experience gradual weight loss [21, 33–35]. Weight loss was greater in the animals fed the high-fat diet, which could be the consequence of the deleterious effect on health caused by the high-fat diet. Previous studies have shown that weight loss in Syrian hamsters towards the end of their lives can be greater when the animals are exposed to damaging factors, such as cigarette smoke [37, 68]. In addition, we observed that the animals fed a high-fat diet had the most severe morphological changes, despite having less accumulation of fat (epididymal fat pad) and lower body weight at the end of the study. Similar results were reported in previous studies that described a paradox in advanced chronic diseases: involuntary weight loss could be related to increased mortality,[69, 70] rather than a protective factor (obesity paradox) [71, 72].

An important aspect of obesity pathophysiology is chronic inflammation [73]. The liver tissue of the animals fed the HFD showed mild inflammation, with no differences between the infected and uninfected animals. In contrast, the livers of the animals fed a balanced diet (infected animals and controls) showed no inflammatory changes. These results once again demonstrate that the HFD was capable of causing more long-term changes than Ad-5 infection alone. Serum globulins were also analyzed. Elevated levels have been related to chronic inflammation [74–77] and pathologies that also present with inflammation, such as NAFLD [64], advanced liver fibrosis [78], or diabetes [76, 79, 80]. Animals that were fed a balanced diet and exposed to Ad-5 in the present study had significantly high levels of serum globulins at follow-up week 22 compared to controls. However, it is striking that animals that were fed an HFD had higher globulin levels than those that were fed a balanced diet, regardless of Ad-5 infection (Table 1). This elevation of the globulin level is most likely associated with a chronic inflammatory state [74–77]. Ad-36 has been shown to elevate IL-6 production, perhaps contributing to the mild chronic inflammation associated with obesity [84]. The lack of measurement of specific inflammation markers in serum and organs other than the liver is a limitation of the present study, and these measurements should be included in future investigations.

The supposition that Ad-5 could potentiate the development of obesity and chronic liver damage is relevant. Adenovirus type 5 is one of the most widely disseminated viruses worldwide. It is a common cause of air-borne infections, as well as of the "common cold" in humans, being responsible for 13% of acute respiratory infections [81]. Therefore, the probable association between obesity and adenovirus type 5 could have great epidemiologic relevance in humans [8]. The majority of studies on adenoviruses as a causal agent of obesity have been carried out with Ad-36. Today, there is no doubt that Ad-9, Ad-31, Ad-36, and Ad-37, and a non-human adenovirus, SMAM1, are linked to increased

adiposity *in vitro* or *in vivo* [1, 3, 4, 82]. The possible role of infection by adenovirus type 5 in relation to the pathogenesis of obesity has not been extensively studied. In 2005, So et al. showed that mice inoculated with Ad-5 had significant weight gain at week 21 (21.8 g for inoculated mice vs. 18.8 g for control mice) [7]. In addition, an epidemiological study associated Ad-5 seropositivity with obesity in children [8].

Multiple mechanisms may condition the pathogenic effects that adenoviruses have on obesity progression. The adipogenic effect has been observed to be a consequence of Ad-36 E4 orf-1 gene expression, because when expression of the gene is blocked, the effect disappears [6]. Increased adiposity is caused by different mechanisms. One of them is the increased expression of peroxisome proliferator-activated receptor-γ, resulting in the differentiation of adult stem cells into adipocytes [4]. Different studies have also shown that Ad-36 infection can produce alterations in serum lipids [85, 86], including an increase in the activity of fatty acid synthase, which converts glucose to fatty acids [4]. Glucose metabolism has also been reported to be altered by Ad-36, demonstrating that cell membrane glucose receptors are increased via the Ras pathway, leading to increased intracellular glucose [4]. Insulin sensitivity has been found to increase, which is being studied in relation to the fight against diabetes [83]. However, there have been no previous reports of an association between Ad-5 infection and serum glucose or lipid elevation, an event that was found in the present study. That is an interesting and previously unreported observation, possibly reflecting a phenomenon associated with significant weight gain caused by Ad-5.

A limitation of the present study was that the affected tissues were not tested for the chronic presence of adenovirus DNA or RNA, and no histologic analysis was performed on muscle or adipose tissues. Future studies that include those aspects will aid in better understanding the pathophysiology of the chronic illnesses caused by adenoviruses.

Further epidemiological studies are needed to evaluate the long-term effects of infection with Ad-5 and other adenoviruses. The present study showed that the effects of Ad-5 infection went beyond that of simply producing obesity. The possible applicability of an anti-adenovirus vaccine (initially against Ad-36) to fight obesity has already been suggested [87], and we can now also suggest that such a vaccine could also prevent or delay severe complications in subjects that present with obesity caused by a high-fat diet. The results of the present study should also be taken into account in evaluating the long-term effects of gene therapy with adenoviral vectors, especially their use in young patients.

In conclusion, Ad-5 infection caused an obesogenic effect in the long term, with adverse metabolic, morphological (hepatic), and functional changes, in an animal model. Despite the fact that a high-fat diet appears to be more harmful in the long term than adenovirus infection alone, infection could increase the severity of harmful effects in individuals with that type of diet. Epidemiologic studies are needed to evaluate the effect of Ad-5 on the genesis of obesity and chronic cardiovascular and liver diseases. The results should also be considered in the evaluation of the long-term effects of gene therapy with adenoviral vectors.

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Compliance with ethical standards

Disclosure of potential conflicts of interest Esteripharma Mexico SA de CV provided support in the form of salaries for author BPM but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. That commercial affiliation does not alter our adherence to the "Archives of Virology" policies on sharing data and materials. The others authors declare that they have no conflict of interest.

Ethical approval The trials complied with the national and international legal and ethical requirements applicable to pre-clinical research. The experimental protocols were approved by the Research Ethics Committee of the School of Medicine of the University of Colima, Mexico (Protocol Number: 14-016). The animals were handled according to institutional guidelines, the Mexican official norm regulating laboratory animal use (NOM-062-ZOO-1999), and the guide for the care and use of laboratory animals issued by the National Academy of Sciences of the United States of America (2011). All animals were euthanized according to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition.

Consent for publication All authors consent to publication.

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