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Original Scientific Article

THE EFFECT OF BEETROOT EXTRACT WITH SILVER NANO PARTICLES ON RUMEN PARAMETERS IN AWASSI LAMBS

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ABSTRACT

Beetroot (*Beta vulgaris*) is a root vegetable with deep red color, rich in vitamins, minerals, fiber and antioxidants, having numerous health benefits. The current study aimed to determine the effect of beetroot extract with silver nanoparticles (AgNPs) on the rumen pH, ammonia, volatile fatty acids (VFAs), total bacterial and protozoal count in Awassi lambs. The beetroot extract was obtained by Soxhlet apparatus. The AgNPs were synthesized from the aqueous beetroot extract. Ten lambs aged (3-6 months) were reared in similar environmental and feeding conditions and were divided into two groups: G1 – fed with AgNPs-Beetroot (AgNPs-BR), orally (3 mg/kg/Bw), daily for 8 weeks; and G2, fed with normal saline. Rumen samples were measured at 2, 4, 6, and 8 weeks after administering AgNPs-BR. Rumen pH was significantly lower in G1 than in G2. The ammonia levels were non-significantly different in the 2nd and the 4th week. However, significant differences were observed at the 6th week. The VFAs, total bacterial, and protozoal count were significantly higher in G1. AgNPs-BR improved rumen function by altering its pH, ammonia, and VFAs concentration, as well as the total bacteria and protozoa count. Therefore, it can be concluded that AgNPs-BR may yield increased economic efficiency in sheep farming.

Key words: beetroot, silver nanoparticles, lambs, rumen, ammonia, volatile fatty acid

INTRODUCTION

The beetroot (*Beta vulgaris*) is a root vegetable plant (1). It has high vitamin B9 content with beneficial effects on cell growth and function, reducing blood vessel and heart diseases. Beets contain nitric oxide having vasodilatory effect, hence it enhances blood perfusion, heart and brain function, and exercise performance (2).

The Fodder beat bulb can be used for sheep fattening. The body weight may be increased by more than 22% (3) by higher energy intake. It has been used in the USA and Europe (4) as an animal feed due to its high sucrose content. It grows during spring in Europe, whereas during winter, it can be used as dried feed (5). It is commonly used in New Zealand as a feed for sheep and cows. It has beneficial effect on regulating acidosis and rumen function (6), and can decrease the risk to rumen acidosis (7). The beetroot extract with silver nanoparticles (AgNPs-BR) can influence the fermentation processes and pH in the rumen by altering the volatile fatty acids (VFA) production necessary for the energy metabolism in ruminants. Maintaining an optimal rumen pH is vital for efficient digestion, acid-base regulation, and microbial activity, which yields high growth performance and feed efficiency in ruminants. The AgNPs-BR may affect the rumen ammonia concentration as a byproduct of protein digestion and microbial protein metabolism, and may have beneficial effect on protein utilization and overall health (8).

The winter feed that includes 65% fodder beet provides adequate nutrition for dried dairy cows, has antioxidant properties, enhances rumen microbes' function, and protects the tissues from oxidative stress. The anti-inflammatory properties of beetroot could provide additional protection during indigestion caused by microbial imbalance or dietary changes. Nanoparticle formulation increases the bioavailability of the active compounds in the beetroot extract, ensuring more efficient utilization by the rumen microbes and the host animal (9).

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The administration of beetroot has been reported to enhance rumen function, growth performance, feed efficiency, and overall health in lambs (10).

The use of silver nanoparticles (AgNPs) in sheep have demonstrated potential antimicrobial properties for multidrug-resistant strains and ability to influence metabolic processes. AgNPs were reported to reduce *Salmonella enteritidis* count, modify the rumen microbiome, reduce methane production, increase nutrient absorption, feed conversion, and productivity in sheep (11).

Our work aimed to study the effect of beetroot AgNPs on rumen pH, total bacterial and protozoal count, ammonia, and volatile fatty acid levels in rams.

MATERIAL AND METHODS

Study design

The study was conducted from May to June 2022 at the animal farm and Laboratory of Public Health, Veterinary Medicine College, University of Baghdad, Iraq.

Ten Iraqi Awassi lambs were housed in semi-open yard under similar atmospheric (18-25 °C, 50-70% humidity) and feeding conditions. Each animal had 1.5–2 square meters of space which was sufficient for free movement, covered with clean and dry sand-based bedding. They were fed with a concentrate diet (2.5% of body weight) consisting of wheat bran 20%, corn 30%, barley 27.5%, wheat 10%, soybeans 10%, calcium carbonate 1%, salt 1%, and dicalcium 0.5%. They had access on pastures (College Fields) for 3-4 h each day. The animals were given green alfalfa, hay, and tap water for two weeks. The feeding time was between 7:00 a.m. and 4:00 p.m. with *ad libitum* straw roughage and tap water (12, 13).

Two equal-weight groups 25–35 kg of five animals were randomly selected and checked for clinical and parasite status (12). The first group (G1) was orally administrated with AgNPs-BR (3 mg/kg/Bw) on daily basis for 8 weeks. The control group (G2) was orally administered with normal saline in the same frequency and duration as G1. The rumen parameters (pH, ammonia, total bacterial count, total protozoal count, and VFA) were measured on 2-week interval, at 2, 4, 6, and 8 weeks after the initial treatment.

The study was approved by the Faculty of Veterinary Medicine at the University of Baghdad, (513/P.G-/Eks/2022).

Chemical reagents, equipment and methods

The beetroot was purchased from local markets (Arak, Iran). The chemical compounds and reagents were provided from Merck (Germany). UV-Vis spectroscopy was used for metallic NPs characterization (14). The UV-Vis spectrum of dispersed nanoparticles (AgNPs) was measured using the UV-Vis spectrophotometer (300 to 800 nm) (UV-Vis Agilent 8541). Fourier Transform Spectroscopy (FT-IR) (PerkinElmer Spectrum two spectrophotometer) has been used to identify the potential of the functional group to reduce the silver ions in the beetroot extract. KBr pellet technique was used for preparation of the extract. The XRD diffraction was used to establish the metallic nature of the particles. The crystalline structure of the AgNPs was confirmed by applying Philips company χ' pert Pro Xray diffract meter by monochromatic Cu kx radiation (λ=1.54 Å) that was maintained at 40 kV, 30 mA. Field emission scanning electron microscopy (FESEM) (VEGAS-TESAH Model) was used to characterize nanoparticle size, shape, and structure. Transmission electron microscopy (TEM) by Philips Company (CM120) was used for other observations.

The beetroot extraction

The Soxhlet apparatus was used for yielding beetroot extract. AgNPs were added according to Wiley and Lee (14). Briefly, beetroot was washed and chopped into small cubes which were mixed with water containing citric acid (0.5%) at ratio 1:3. The mixture was vortexed in water bath (WiseBath, South Korea) for 60 min at 80 °C. Then, it was cooled to 25 °C and filtered. The beetroot extract was concentrated in evaporator (Heidolph, Germany) at 50 °C (15). Beetroot powder (10 g) was added into 100 mL of distilled water and was boiled, stilled for 5 min, and filtered with the Whatman No.1 paper. It was centrifuged at 4,000 rpm and stored in the refrigerator at 4 °C.

Preparation of beetroot with silver nanoparticles

Silver nitrate ($AgNO_3$) 10 mM (1.7 g) 100 mL was prepared as a stock solution. Several diluted solutions were made (1, 1.5, 2, 2.5, 3, and 5 mM). By adding the beetroot, the solution color changed from ruby red to brown due to the interaction of silver and the production of AgNPs (16) (Fig. 1).

A volume of 0.5 mL of beetroot extract was mixed with 5 mL of $AgNO_3$ 1 mM (169. 87 mg) and was vortexed for 30 min in different pH conditions (2, 4, 5, 6, 7, 8 and 10) in order to determine the optimal pH, HCl, and NaOH 0.1 M concentrations. The pH was measured with an EDT GP 353 ATC pH meter. In the next step, it was centrifuged (30 min) at 4,000 rpm. These steps were repeated for different extract volumes (0.1, 0.2, 0.25, 0.5, 1.5 and 2 mL), solute concentrations (1, 1.5, 2, 2.5, 3 and 5 mM), and centrifuging time (10, 20, 40, 60, 90 and 120 min).

After determining the optimal extraction conditions, the AgNPs were synthesized, and the sediment was dried in an oven at 25 °C after centrifugation. UV–Vis, FT-IR, XRD, FESEM and TEM were used to characterize the dried particles.

The extract (25 mL) contained 0.008 g of AgNPs. The absorption of 1 mL of extract with 0.1 mL of NaBH4 0.01 M and 3 mL methylene blue 5-10 M was analyzed and measured at different times. The removal of methylene blue by NaBH4 in the presence of the AgNPs was monitored using the UV spectrum at 664 nm.

Rumen content parameters

Ammonia concentration - After collection, rumen fluid (10 mL) was frozen in a 50 mL tube with 5 mL of HCL at -20 °C. After thawing, the samples were centrifuged at 2,500 rpm for 10 min and the yellow fluid precipitate was removed. MgO and $CaCl_2$ (0.5 g) were added 0.5 mL ruminal solution. The ammonia level was measured by adding HCl (0.05%) until the red methylene changed to purple color. Ammonia concentration was expressed in mg/dL (17, 18).

pH – It was measured immediately after the rumen content collection. pH value was expressed as pH meter scale.

Total bacterial count – Rumen fluid content (1 mL) was placed in a plastic tube with distilled water (8 mL) and was transported to the laboratory. After additional dilution ($x10^6$), a 1 mL sample was placed on microscope slide and was observed on a light microscope. The rumen fluid was placed on selective and non-selective media and the number of colony-forming units (CFUs) were counted to estimate the number of viable bacteria. This method detects bacteria that are culturable and may represent a small proportion of the total microbial population (19). Total bacterial count was expressed as $CFU\backslash 10^6$.

Total protozoal count - The samples were aliquoted in two burettes of one litter. Glucose (0.5 gr) was added in each burette and were incubated at 39 °C for 60 min. The microorganisms, including protozoa, precipitated at the burette's bottom (20). They were transferred into a 50 mL tube, centrifuged at 100 rpm for ten min, and then washed with Coleman's buffer (21). The pellet was diluted with distilled water (1:10) and was left to still for 120 min at 25 °C before it was observed under light microscope (22). Total protozoal count was expressed as 10^6/mL.

Volatile fatty acid (VFA) – They were measured with gas chromatography method. Volatile fatty acids (VFA) were expressed as mmol/L).

Statistical analysis

The data are represented as mean ± SD. Two-way ANOVA and LSD were used for comparison at probability level of $p<0.05$. SPSS software 2022 was used for the statistical analysis (23).

RESULTS

Rumen pH was significantly lower in G1 during the entire experimental period. However, no significant differences were observed between the experimental periods (Fig. 1).

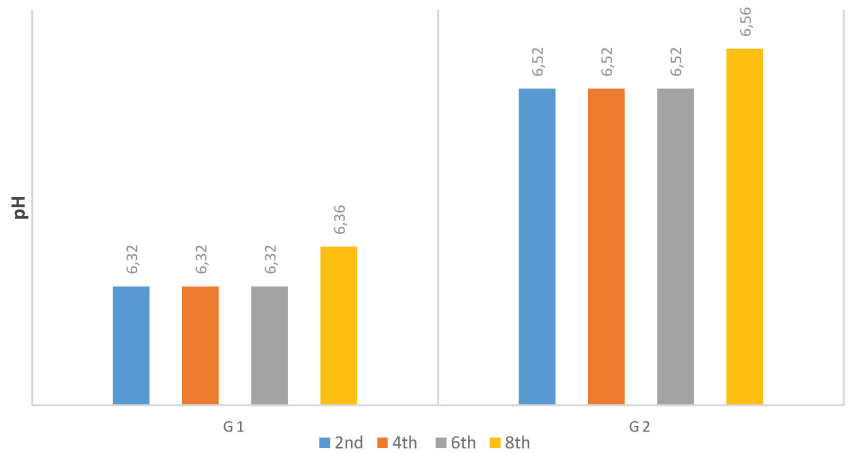

Figure 1. Rumen pH. Columns represent weeks of sampling. G1 (treatment group) - orally administrated with beetroot extract with silver nanoparticles (3 mg/kg/Bw), daily for 8 weeks; G2 (control group) - orally administered with normal saline in the same frequency and duration as G1. Columns with different capital letters between the groups are significantly different, and those with different small letters are significantly different between the weeks of sampling in the same group

The ammonia level of rumen liquid was significantly higher in G1 on the 6th week. The same group had higher values for the other sampling periods, but with no significant difference (Fig. 2).

The total bacterial count in the rumen content was significantly higher in G1 during the entire experimental period. Furthermore, these values had an increasing trend ($p<0.05$) during the experimental period (Fig. 3).

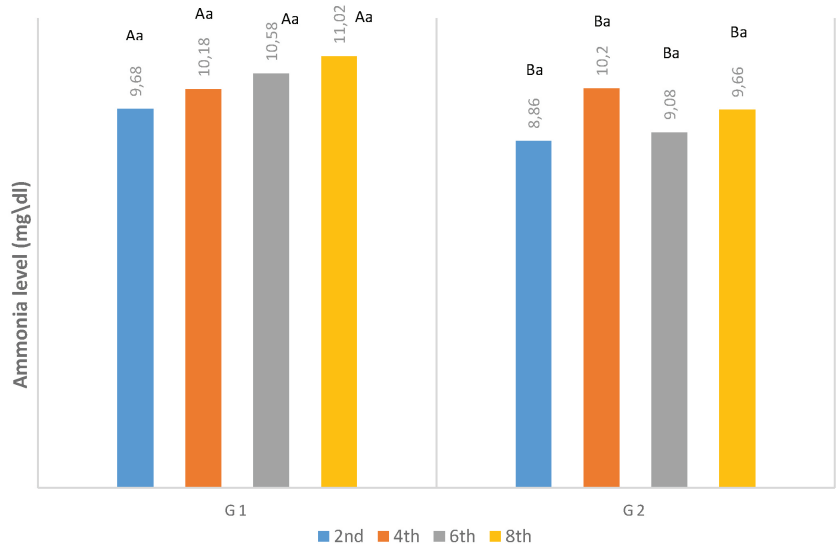

Figure 2. Ammonia level in the rumen content (mg/dl). Columns represent weeks of sampling. G1 (treatment group) - orally administrated with beetroot extract with silver nanoparticles (3 mg/kg/Bw), daily for 8 weeks; G2 (control group) - orally administered with normal saline in the same frequency and duration as G1. Columns with different capital letters between the groups are significantly different, and those with different small letters are significantly different between the weeks of sampling in the same group

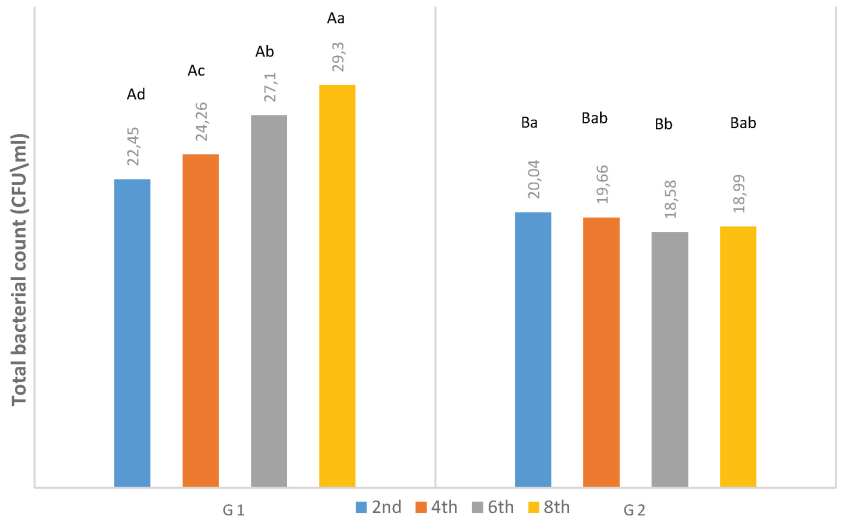

Figure 3. Total bacterial count in rumen content (CFU/ml). Columns represent weeks of sampling. G1 (treatment group) - orally administrated with beetroot extract with silver nanoparticles (3 mg/kg/Bw), daily for 8 weeks; G2 (control group) - orally administered with normal saline in the same frequency and duration as G1. Columns with different capital letters between the groups are significantly different, and those with different small letters are significantly different between the weeks of sampling in the same group

Total protozoal count in the rumen content was significantly higher in G1 at the 2nd, 6th, and 8th week. The increasing trend was significantly different between the 2nd-4th week and 6th–8th weeks, however no significant differences were observed between 2nd and 4th week, as well as between 6th and 8th week (Fig. 4).

Total volatile fatty acids levels in the rumen liquid were significantly higher in G1 during the entire experimental period and had an increasing trend ($p<0.05$). The G2 group had non-significant increasing trend (Fig. 5).

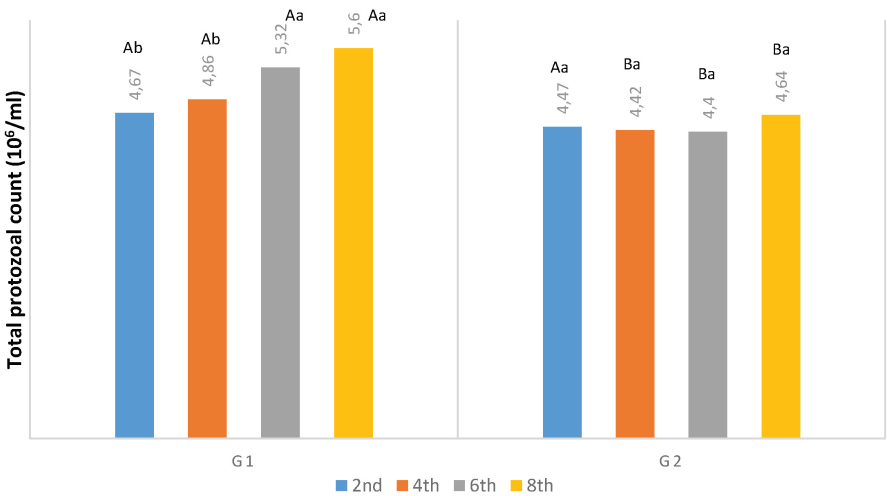

Figure 4. Total protozoal count in the rumen content (10^6/ml). Columns represent weeks of sampling. G1 (treatment group) - orally administrated with beetroot extract with silver nanoparticles (3 mg/kg/Bw), daily for 8 weeks; G2 (control group) - orally administered with normal saline in the same frequency and duration as G1. Columns with different capital letters between the groups are significantly different, and those with different small letters are significantly different between the weeks of sampling in the same group

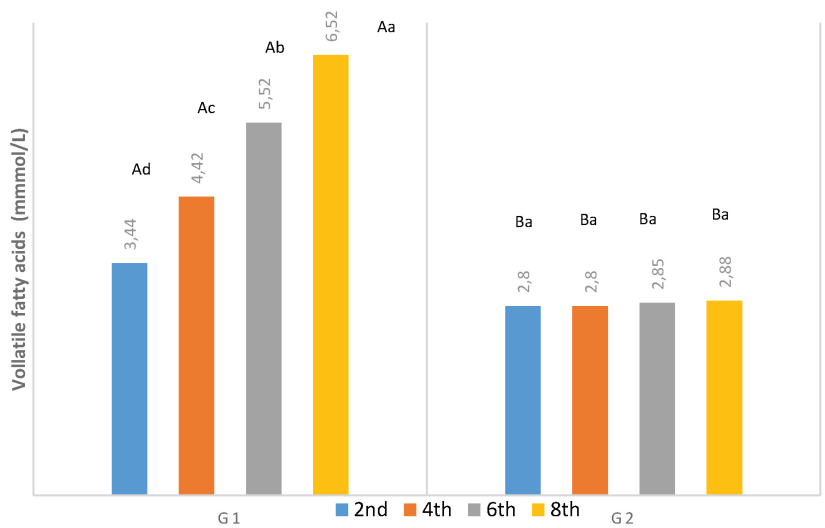

Figure 5. Volatile fatty acid in the rumen content (mmol/L). Columns represent weeks of sampling. G1 (treatment group) - orally administrated with beetroot extract with silver nanoparticles (3 mg/kg/Bw), daily for 8 weeks; G2 (control group) - orally administered with normal saline in the same frequency and duration as G1. Columns with different capital letters between the groups are significantly different, and those with different small letters are significantly different between the weeks of sampling in the same group

DISCUSSION

The current study is the first report on the effect of beetroot extract with AgNPs on rumen parameters in Iraqi Awassi lambs. AgNPs showed specific chemical and physical properties, rendering high solubility, stability, and reactivity. Rumen pH was significantly lower in the group treated with AgNPs-BR.

It has been previously reported that beetroot supplementation in the feed for cows reduced rumen pH from 6.37 to 5.94, rumen acidosis occurrence was lowered, and antioxidant status was enhanced (24). Ruminants that consume beetroot daily, have enhanced mastication, rumination, and overall, improved ruminal activity and function (25). These effects may be attributed to the beetroot ingredients (10).

The current study demonstrated that rumen ammonia level was higher in the group that was supplemented with AgNPs-BR. Rumen microorganisms can transfer the non-protein nitrogen (NPN) to the ammonia, generating ketoacids and amino acids. The urease converts urea to ammonia (26, 27). Another study, reported similar findings with higher rumen ammonia levels in ewes fed with beetroot compared to the control (28). This effect can be attributed to the high protein content (16.21%) in the beetroot, which undergoes microbial utilization and transformation into ammonia as byproduct (12, 29).

The current study demonstrated significantly higher rumen bacteria count in G1 throughout the whole experimental period which is supported by the findings of a similar study with dairy cows fed with fresh beetroot (10). Also, the total protozoa count was significantly higher in G1, with exception of the second week. Protozoa comprise more than 50% of the rumen biomass and have a role in catabolizing rumen carbohydrates (30) and reducing rumen fungus and bacteria, hence decreasing the epithelial shedding (31, 32).

AgNPs have potent bactericidal properties due to their ability to damage bacterial cell walls. However, they may have an unequal bactericidal effect in sheep rumen. The rumen contains a diverse microbial population, including beneficial and harmful bacteria. AgNPs inhibit or kill certain pathogenic strains leaving the beneficial bacterial species unaffected. In low concentrations they may stimulate the growth of certain bacteria by acting as a stress factor. Some bacteria may have adaptive survival mechanisms that can be triggered by the presence of nanoparticles. This effect is called 'hormesis', where low levels of a toxic agent induce a beneficial biological response. The bactericidal action of AgNPs can disrupt the balance between different bacterial groups in the rumen, creating an ecological gap that allows the surviving bacteria to proliferate more rapidly. This may lead to an overall increase in the total bacterial population as the community readjusts to the altered environment. The rumen's complex and anaerobic environment could affect AgNPs' behavior, rendering them less effective in killing bacteria. Organic matter binding may reduce or neutralize their activity. However, by enhancing the fermentation processes, AgNPs may yield higher nutrient availability for the bacteria, leading to a population rise (33, 34, 35). This increase in bacterial concentration does not necessarily indicate a failure of the AgNPs to exhibit bactericidal activity but rather a complex interaction between the nanoparticles and the rumen's unique microbial ecosystem (36, 37).

The total VFA level in the rumen showed significant increasing trend in G1 throughout the experimental period. Easily fermentable carbohydrates in feeds, such as the beetroot pulp, are a substrate for generation of VFA by the rumen anaerobic processes. The VFA buildup may cause fluctuations in the pH level (38). Physically pulverized fodder may promote microbial adherence to ingested material and may enhance the motility and passage of the rumen digested contents. Beetroot promotes chewing, mastication, and salivation, providing balanced pH environment and increased rumen absorption of VFAs (39). However, buildup of VFA may decrease ruminal pH after extensive consumption of beetroot. Recent research has documented the time-dependent changes in rumen fermentation during beetroot consumption (40).

CONCLUSION

Beetroot extract with silver nanoparticles improved rumen function in Awassi lambs by altering rumen pH, ammonia, VFA, total bacterial, and protozoal count. These effects may promote higher digestibility, motility, and absorption of feed compounds, yielding higher economic efficiency in sheep farming.

CONFLICT OF INTEREST

The authors declare that they have no financial or non-financial conflict of interest regarding authorship and publication of this article..

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AUTHORS' CONTRIBUTION

TND conducted the experiment, both on farm and in the lab, made the statistical analysis, and wrote the manuscript.

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