JOURNAL OF NUTRITION FASTING AND HEALTH

Inhibitory Effect of Nano-gel/Emulsion of Chitosan Coating Incorporated with *Ziziphora Clinopodioides* Essential Oil and Nisin on *Escherichia Coli* O_{157}:H_7 Inoculated in Beef at Cold Storage Condition

Asghar Azizian[1], Saeid Khanzadi[*1], Mohammad Hashemi[2], Mohammad Azizzadeh[3]

1. Department of Food Hygiene and Aquatics, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran.
2. Department of Nutrition, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
3. Department of Clinical Science, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran.

ARTICLEINFO

Article type:
Research Paper

Article History:
Received: 30 Apr 2019
Accepted: 29 May 2019
Published: 25 Jun 2019

Keywords:
Chitosan
Functional coating
Nano technology
Beef
Escherichia coli O_{157}:H_7

ABSTRACT

Introduction: This study was conducted to evaluate the influence of chitosan Nano-gel/emulsion coating functionalized by *Ziziphora clinopodioides* essential oil (ZCEO) and nisin on growth inhibition of *Escherichia coli* O_{157}: H_7 inoculated in beef samples during 16 days in cold storage condition (4˚C).

Methods: Beef sample was divided into six groups after inoculation of *E. coli* O_{157}:H_7. Treatments including control (no coating), chitosan 2%, sonicated chitosan 2%, Nano-emulsion of chitosan coating containing ZCEO (0.5%), Nano-gel of chitosan coating containing nisin (200 IU/g), Nano-emulsion of chitosan coating containing ZCEO (0.5%) and nisin (200 IU/g) were stored at refrigeration temperature and bacterial count were performed on days: 0, 1, 2, 4, 8, 12 and 16. Data were analyzed using repeated measure ANOVA and Bonferroni post hoc tests. **Results:** Result indicated a significant reduction in *E. coli* O_{157}:H_7 count in all treatments when compared to control group and the highest inhibitory activity was observed in chitosan Nano-emulsion coating containing ZCEO (0.5%) and nisin (200 IU/g).

Conclusion: Accordingly, it is suggested that chitosan Nano-emulsion coating with ZCEO and nisin practically be applied in beef to increase its safety against pathogenic bacteria especially *E. coli* O_{157}: H_7.

► *Please cite this paper as*:
Azizian A, Khanzadi S, Hashemi M, Azizzadeh M. Inhibitory Effect of Nano-gel/Emulsion of Chitosan Coating Incorporated with Ziziphora Clinopodioides Essential Oil and Nisin on Escherichia Coli O157:H7 Inoculated in Beef at Cold Storage Condition. J Nutrition Fasting Health. 2019; 7(2): 103-109. DOI: 10.22038/jnfh.2019.40026.1192

Introduction

Beef has the third global position among meat consumers that accounts for approximately 25% of the meat needs. The ingredients of beef and relevant products are proteins, lipids, and importantly moisture content, thus predisposing the growth of different species of microorganisms as “natural media” (1). The pathogenic bacteria after infecting beef and its products proliferates rapidly in such excellent nutrients; among which *Escherichia coli* O_{157}:H_7 is a bacterial agent to contaminate the beef and its products (2), it can produce entero-haemorrhagic toxins and deferent virulence factors responsible for mild to severe or bloody and painful diarrhea and other adverse effects (3). Moreover, these bacteria are able to spread different kinds of food owing to surface contamination basically in post-processing phases (4).

Accordingly, the recent studies are looking for naturally occurring food preservatives to prolong the shelf life of food products without any complications, establishing important field of study for researchers and producers (5).

* *Corresponding author:* Saeid Khanzadi, Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. Tel: +985138805610; Fax: +985138763852; Mobile: Email: Khanzadi@um.ac.ir

Edible coatings and films have attracted further attention because of their ability to carry food additives, including antimicrobials, antioxidants, colors, and flavors, as well as to keep high doses of such agents on the food surface (4). In this context, one of the preservatives widely used in food packaging is chitosan owing to capability of forming film with antimicrobial activity (6). A lot of antimicrobial agents are available to control the growth of different bacteria, fungi and yeast in food products using the chitosan coating (7), with various methods of applying preservatives, such as directly augmentation into food (8), in the form of sachets into packages (9), direct application on the food surfaces (6), and incorporated with packaging materials (10).

Essential oils (EOs) contain various phenolic compounds, including flavonoids and phenolic acids, with antimicrobial nature against to a broad spectrum of microorganisms (4, 11). *Ziziphora clinopodioides* essential oil (ZCEO) is a widely used spice in food preservative owing to potent antioxidant and antimicrobial activities, and also as carminative, appetitive, stomach tonic, antiseptic and expectorant (12), In addition to EOs, other natural antimicrobial agents known as generally recognized as safe (GRAS), including nisin, have been recently at the center of attention (12, 13).

Nisin is a more prevalent bacteriocin synthesized by strains of *Lactococcus lactis* and *Streptococcus uberis*, which is administered as an additive and preservative in foods like cheese (14), beef (12, 15) and chicken (16). It is reportedly a strong substance to eliminate a vast spectrum of gram-positive bacteria (17). It is also can be functionalized and coupled with different EOs (12, 17).

Nano-emulsions can be specifically prescribed for food products because of their unique features, including ease of preparation, high-grade functions and fine particle size, which provide enhanced interactions of active compounds and bio membranes, and their transfer (18). The Nano-emulsions are produced by multiple approaches; for example, low-energy and high-energy techniques (18). One of the high-energy methods is ultrasonic emulsification that can be effectively applied to prepare Nano-emulsions having small droplet diameters and low size distributions (19). The emulsion-based systems developed by food-grade components are easily distributed in food to control the growth of various existing microorganisms (20).

There have been many studies concentrating on the application of edible coatings and films in food; but, few studies have used chitosan as coating containing Nano-particles in food products (21, 22), and there have been no studies regarding effect of chitosan Nano-gel/emulsion coatings containing natural antimicrobials against food-born pathogenic in beef. Accordingly, the present study was designed to investigate the inhibitory activity of nisin and ZCEO-containing chitosan Nano-gel/emulsion alone and in combination on the growth of *E. coli* O_{157}:H_7 inoculated in beef samples at 4˚C.

Materials and methods

Materials

The ZCEO was prepared from Iranian institute of medicinal plants, Karaj, Alborz province, Iran. Low molecular weight (LMW; 1.03×10^5) chitosan with degree of deacetylation 91% was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). All media cultures were prepared from Merck (Darmstadt, Germany). Nisin and all other reagents with analytical grade were prepared from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). *E. Coli* O_{157}:H_7 (NCTC 12900) was obtained from the department of food hygiene, faculty of veterinary medicine, Ferdowsi university of Mashhad, Mashhad, Iran.

Preparation of E. coli O_{157}:H_7

It was cultured in 9 mL of brain heart infusion (BHI) broth, incubated at 37°C for 24 hours, and then re-incubated for another 18 hours at 37°C. The used bacterial suspension was obtained from 18-h culture (23) to prepare 0.5 McFarland turbidity standard (containing 1.5×10^8 CFU/mL) and diluted (1:10) to density of 1.5×10^7 CFU/mL (23).

Preparation of chitosan Nano-gel/emulsion coating containing ZCEO and nisin

The chitosan powder (2% w/v) (in 5 sterile container) was dissolved in sterile distilled water containing acetic acid (1%, w/v), and glycerol (1.5% v/v) as a plasticizer, to prepare the solutions of chitosan, followed by constantly stirring for 10-15 min to obtain clear solutions. On the other hand, 50 mg of nisin was dissolved

in 5 ml of hydrochloric acid (0.02 mol/L) to provide stock solution of nisin, and then the ZCEO (0.5% $_{v/w}$) and nisin (200 IU/g) were added to the chitosan solutions. The ZCEO was poured in chitosan solutions in the presence of tween 80 (0.2% w $_{ZCEO}$), as an emulsifier, followed by stirring for 30 min to become uniform, stable, and clear emulsion. Then Chitosan Nano-gel/emulsion was formulated in a basis of protocol introduced by Ghosh et al. (2013) with slight modification. Coating solutions were subjected to ultraturrax for 3 min at 3000 rpm and ultrasonic emulsification sonicator (50 ˚C, pulse; 45s and rest; 15s) for 6 min (19). Particle size was measured by DLS device as well.

Preparation of the beef samples and inoculation of the bacteria

The fresh beef was purchased from a local butchery of Mashhad, Iran, in summer 2018, and immediately was transmitted to the laboratory. The blood and slime of beef samples were removed by washing and then they were dried, sectioned into pieces (10 g), sprayed by ethanol (70% v/v), and burnt to remove the surface microorganisms (11). Each side of samples were separately inoculated by 50 µL of bacterial suspension (1.5×10^8 CFU/mL) using micropipettes to achieve a final concentration of about 10^6 CFU/g (4, 11).

Preparation of treatments

The inoculated samples were divided into six groups (Table 1) and then were treated by immersing in chitosan Nano-gel/emulsion coatings for 1 minute, drained for 15 minutes, and stored at 4°C for 16 days. Finally, the analysis was performed on days of 0, 1, 2, 4, 8, 12 and 16 during storage (4).

Table 1. List of treatments in the present study

No	Treatment	Description
1	CON	Control: Samples without any coating
2	Ch 2%	Samples coated with chitosan 2%
3	Nano Ch 2%	Samples coated with sonicated chitosan 2%
4	Nano Ch+ ZCEO	Samples coated with Nano-emulsion of chitosan containing 0.5% (w/v) ZCEO
5	Nano Ch+ Nisin	Samples coated with Nano-gel of chitosan containing nisin 200IU/g
6	Nano Ch+Nisin+ ZCEO	Samples coated with Nano-emulsion of chitosan containing 0.5% (w/v) ZCEO and nisin 200IU/g

***Enumeration of E. coli* O_{157}:H_7**

For enumeration of the inoculated bacteria, the samples (10 g) were brought to a final volume of 90 mL with 0.1% sterile peptone water. Then, a stomacher (Seward Medical, London, UK) was used for two minutes to homogenize the samples. After the preparation of decimal dilutions, 10 µL (drops method) (4, 24) of serial dilutions of homogenates were cultured onto Cefixime Tellurite Sorbitol MacConkey (CT-SMAC) agar (4, 25) and incubated at 37°C for 24 hours.

Statistical analysis

All results were presented as mean ± standard deviation (SD) of three measurements and statistical analysis was performed using SPSS ver. 21 software (SPSS, Inc. Chicago, USA) using repeated measure ANOVA, followed by Bonferroni post- hoc test and Dunnette T3 tests. $P< 0.05$ was considered as the significance level.

Results and discussion

Nano-gel/emulsion characterization

Table 2 represent the mean droplet size in the Nano-gel/emulsion and their PDI in different groups. As shown in Table 2, the mean droplet size and PDI also decreased after preparation of different Nano-gel/emulsion coatings. The mean droplet size decreased in the sonicated chitosan coating in comparison with that of chitosan coating (non-sonicated). Identical results were

demonstrated in previous studies (26); they observed that the droplet size of the Nano-emulsion coated with chitosan decreased. The highest particle size was observed in chitosan coating (2194 nm), and the lowest was observed in Nano ch+nisin + ZCEO (401.3 nm). Other researchers fabricated Nano-emulsion by the incorporation of EOs and antibacterial agents in the biopolymer solutions such as sodium alginate (27) and chitosan (28) prior to sonication process. All of these researchers reported larger droplet sizes (less than 1000 nm), than that obtained in this research (lowest was 401.3 nm).

Table 2. Comparison of Lipid Profiles in Participants before and after Intervention

Group	z-average (d.nm)	PDI
Ch 2%	2194	0.300
Nano Ch 2%	904.4	0.332
Nano Ch+ ZCEO	602.9	0.292
Nano Ch+ Nisin	595.8	0302
Nano Ch+Nisin+ ZCEO	401.3	0.406

As shown in Table. 2, the PDI also decreased after Nano-gel/emulsion fabrication. The PDI obtained in this research was less than 0.450 in different groups. Noori et al. (2018), observed that the PDI was recorded as 0.584 for GEO emulsion and decreased to 0.222 when conventional Nano-emulsion was prepared. Also, some other researchers reported that the PDI decreased after sonication (in all of them, PDI less than 0.500 reported) (21, 29). The lower PDI of Nano-emulsion approved the efficiency of ultra-sonication method in formation of a uniformly size distributed Nano-emulsion (21).

***Enumeration of E. coli* O_{157}:H_7**

Table 3 represents the effect of different treatments on growth of *E. Coli* O_{157}:H_7 during 16 days of storage. The initial count of *E. Coli* O_{157}:H_7 was 6.55 ± 0.05 log CFU/g in CON samples, although, it was lower than CON samples in other treatments, which it may be due to the primary effect of treatments on growth of *E. Coli* O_{157}:H_7. The growth of *E. coli* O_{157}:H_7 decreased at 4˚C in all treatments; similar results were obtained in previous studies (22). The maximum bacterial count was observed in CON samples (on 16^{th} day of storage; 4.45 ± 0.13 log CFU/g), and the minimum count was observed in Nano Ch+Nisin+ ZCEO samples (on 8^{th} day of storage; 3.15 ± 0.21, 12^{th} and 16^{th} <3 log CFU/g), respectively. Similarly, Shahbazi et al. (2015a) found that ZCEO and nisin could decrease the *E. coli* O_{157}:H_7 growth in culture media especially when they were used in combination.

Table 3. Changes in bacterial count (Log CFU/g) of beef samples inoculated with E. coli O157H7 during storage (M ± SD)

Day	CON	Ch 2%	Nano Ch 2%	Nano Ch+ Nisin	Nano Ch+ ZCEO	Nano Ch+Nisin+ ZCEO
0	6.55 ± 0.05	5.39 ± 0.13	5.22 ± 0.11	5.12 ± 0.11	4.84 ± 0.15	4.73 ± 0.15
1	6.35 ± 0.08	4.86 ± 0.12	4.47 ± 0.11	4.40 ± 0.09	4.31 ± 0.01	4.01 ± 0.07
2	6.09 ± 0.11	4.53 ± 0.08	4.25 ± 0.03	4.24 ± 0.13	3.89 ± 0.05	3.77 ± 0.09
4	5.76 ± 0.07	4.36 ± 0.05	4.10 ± 0.09	4.05 ± 0.07	3.68 ± 0.09	3.56 ± 0.09
8	5.33 ± 0.08	4.24 ± 0.07	3.81 ± 0.11	3.75 ± 0.13	3.36 ± 0.09	3.15 ± 0.21
12	4.89 ± 0.30	4.11 ± 0.17	3.67 ± 0.15	3.63 ± 0.17	3.12 ± 0.16	ND
16	4.45 ± 0.13	3.90 ± 0.04	3.38 ± 0.08	3.34 ± 0.20	ND	ND

ND: Non detected (ND<103)

The mean reduction rate of *E. coli* O_{157}:H_7 count in various treatments is shown in Table 4, so that the maximum reduction rate was related to Nano ch+nisin + ZCEO (2.81 log CFU/g) and Nano ch+ ZCEO (2.31 log CFU/g), when they were compared to CON. As it can be seen in Table 4, the use of combinational antimicrobial agents, is more effective against microbial growth than their individual use. Several previous studies (Shahbazi et al., 2015; Ehsani et al., 2014; Raeisi et al., 2016) confirmed the above finding; nevertheless, they may have antagonistic, synergistic, or additive effects according to the type of antimicrobial agent and microorganism. Heretofore this is the first study which reported that the application of coating treatments could eliminate the bacterial count to level of non-detectable (10^3 CFU/g). As mentioned it is may be due to use of coating solutions containing ZCEO and nisin as Nano-emulsions.

Table 4. Average reduction rate of the *E. coli* $O_{157}H_7$ counts (Log CFU/g) among treatments when compared each other during study period

Mean Difference I-J Group (I)	Group (J) CON	Ch 2%	Nano Ch 2%	Nano Ch+ Nisin	Nano Ch+ ZCEO	Nano Ch+Nisin+ ZCEO
CON		1.14*	1.50*	1.55*	2.31*	2.81*
Ch 2%			0.35*	0.40*	1.17*	1.73*
Nano Ch 2%				0.05	0.81*	1.38*
Nano Ch+ Nisin					0.76*	1.32*
Nano Ch+ ZCEO						0.56*

Conclusion

According to the results obtained from the present study, Nanochitosan coating was more effective than chitosan coating in eliminating E. coli O157:H7 in beef samples at 4°C. The Nano-emulsion of chitosan combined with ZCEO and nisin (Nano ch+nisin + ZCEO) indicated the highest inhibitory effect on the growth of E. coli O157:H7 inoculated in beef samples at 4°C. The treatments had an acceptable effect on high doses (106 CFU/g) of E. coli O157:H7 and could effectively accelerate its reduction rate in contaminated beef samples stored in at 4°C. These antimicrobial agents loaded in sonicated chitosan coating could significantly reduce the growth of E. coli O157:H7. Accordingly, given the preference of producer and consumer for using natural food additives, we recommend the administration of Nano-emulsion of chitosan enriched with ZCEO and nisin in beef to significantly control the pathogenic bacteria for achieving adequate safety.

Acknowledgments

It is essential to acknowledge the Ferdowsi University of Mashhad, Mashhad, Iran, for funding (grant No; 3/46031) the present study and Mrs. S. Khajenasiri for cooperating in the current research.

Conflict of interests

There is no conflict of interests in this study.

References

1. Cui H, Yuan L, Lin L. Novel chitosan film embedded with liposome-encapsulated phage for biocontrol of Escherichia coli O157: H7 in beef. Carbohydr polym. 2017; 177: 156-64.
2. Zarei M, Basiri N, Jamnejad A, Eskandari MH. Prevalence of Escherichia coli O157: H7, Listeria monocytogenes and Salmonella spp. in beef, buffalo and lamb using multiplex PCR. Jundishapur J Microbiol. 2013; 6(8): e7244.
3. Djenane D, Aïder M, Yangüela J, Idir L, Gómez D, Roncalés P. Antioxidant and antibacterial effects of Lavandula and Mentha essential oils in minced beef inoculated with E. coli O157: H7 and S. aureus during storage at abuse refrigeration temperature. Meat Sci. 2012; 92(4): 667-74.
4. Sharifi F, Khanzadi S, Hashemi M, Azizzadeh M. Control of Listeria monocytogenes and Escherichia coli O157: H7 inoculated on fish fillets using alginate coating containing lactoperoxidase system and Zataria multiflora boiss essential oil. Journal of Aquatic Food Product Technology. 2017; 26(9): 1014-21.
5. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol. 2004; 94(3): 223-53.
6. Park SI, Marsh KS, Dawson P. Application of chitosan-incorporated LDPE film to sliced fresh red meats for shelf life extension. Meat Sci. 2010; 85(3): 493-9.
7. Dutta PK, Tripathi S, Mehrotra GK, Dutta J. Perspectives for chitosan based antimicrobial films in food applications. Food chemistry. 2009; 114(4): 1173-82.
8. Shahidi F, Arachchi JKV, Jeon YJ. Food applications of chitin and chitosans. Trends in food science & technology. 1999; 10(2): 37-51.
9. Coma V. Bioactive packaging technologies for extended shelf life of meat-based products. Meat scie. 2008; 78(1-2): 90-103.
10. Basher A. Color Improvement and Shelf Life Extension of Fresh Red Meats Packaged with Chitosan Coated Films. Archived Dissertations. 2000; 801.
11. Raeisi M, Tajik H, Razavi RS, Maham M, Moradi M, Hajimohammadi B, et al. Essential oil of tarragon (Artemisia dracunculus) antibacterial activity on Staphylococcus aureus and Escherichia coli in culture media and Iranian white cheese. Iran J Microbiol. 2012; 4(1): 30-4.
12. Shahbazi Y, Shavisi N, Mohebi E. Effects of Ziziphora clinopodioides Essential Oil and Nisin, Both Separately and in Combination, to Extend Shelf Life and Control E scherichia coli O 157: H 7 and S taphylococcus aureus in Raw Beef Patty during Refrigerated Storage. J Food Saf. 2015; 36(2): 227-36.
13. Yoon JI, Bajpai VK, Kang SC. Synergistic effect of nisin and cone essential oil of Metasequoia glyptostroboides Miki ex Hu against Listeria monocytogenes in milk samples. Food Chem Toxicol. 2011; 49(1): 109-14.
14. Al-Holy M, Al-Nabulsi AA, Osaili T, Ayyash MM, Shaker RR. Inactivation of Listeria innocua in brined white cheese by a combination of nisin and heat. Food Control. 2012; 23(1): 48-53.
15. Solomakos N, Govaris A, Koidis P, Botsoglou N. The antimicrobial effect of thyme essential oil, nisin, and their combination against Listeria monocytogenes in minced beef during refrigerated storage. Food Microbiol. 2008; 25(1): 120-7.
16. Economou T, Pournis N, Ntzimani A, Savvaidis IN. Nisin–EDTA treatments and modified atmosphere packaging to increase fresh chicken meat shelf-life. Food Chem. 2009; 114(4): 1470-6.
17. Gao M, Feng L, Jiang T, Zhu J, Fu L, Yuan D, et al. The use of rosemary extract in combination with nisin to extend the shelf life of pompano (Trachinotus ovatus) fillet during chilled storage. Food Control. 2014; 37: 1-8.
18. Moghimi R, Ghaderi L, Rafati H, Aliahmadi A, McClements DJ. Superior antibacterial activity of

nanoemulsion of Thymus daenensis essential oil against E. coli. Food Chem. 2016; 194: 410-5.
19. Ghosh V, Mukherjee A, Chandrasekaran N. Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity. Ultrason Sonochem. 2013; 20(1): 338-44.
20. Donsì F, Annunziata M, Sessa M, Ferrari G. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. Journal of Biotechnology. 2010; 44(9): 1908-14.
21. Noori S, Zeynali F, Almasi H. Antimicrobial and antioxidant efficiency of nanoemulsion-based edible coating containing ginger (Zingiber officinale) essential oil and its effect on safety and quality attributes of chicken breast fillets. Food control. 2018; 84: 312-20.
22. Severino R, Ferrari G, Vu KD, Donsì F, Salmieri S, Lacroix M. Antimicrobial effects of modified chitosan based coating containing nanoemulsion of essential oils, modified atmosphere packaging and gamma irradiation against Escherichia coli O157: H7 and Salmonella Typhimurium on green beans. Food Control. 2015; 50: 215-22.
23. Ehsani A, Hashemi M, Naghibi SS, Mohammadi S, Khalili Sadaghiani S. Properties of Bunium persicum essential oil and its application in Iranian white cheese against Listeria monocytogenes and Escherichia coli O157: H7. J Food Saf. 2016; 36(4): 563-70.
24. Shokri S, Ehsani A, Sedigh Jasour M. Efficacy of lactoperoxidase system-whey protein coating on shelf-life extension of rainbow trout fillets during cold storage (4 C). Food Bioproc Tech. 2014; 8(1): 54-62.
25. Shin JH, Chang S, Kang DH. Application of antimicrobial ice for reduction of foodborne pathogens (Escherichia coli O157: H7, Salmonella Typhimurium, Listeria monocytogenes) on the surface of fish. J Appl Microbiol. 2004; 97(5): 916-22.
26. Li X, Qi J, Xie Y, Zhang X, Hu S, Xu Y, et al. Nanoemulsions coated with alginate/chitosan as oral insulin delivery systems: preparation, characterization, and hypoglycemic effect in rats. Int J Nanomedicine. 2013; 8: 23-32.
27. Alparslan Y, Baygar T. Effect of chitosan film coating combined with orange peel essential oil on the shelf life of deepwater pink shrimp. Food Bioproc Tech. 2017; 10(5): 842-53.
28. Wu C, Wang L, Hu Y, Chen S, Liu D, Ye X. Edible coating from citrus essential oil-loaded nanoemulsions: physicochemical characterization and preservation performance. RSC Adv. 2016; 6(25): 20892-900.
29. Hosseinnia M, Khaledabad MA, Almasi H. Optimization of Ziziphora clinopodiodes essential oil microencapsulation by whey protein isolate and pectin: A comparative study. Int J Biol Macromol. 2017; 101: 958-66.