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Ozone treatment as a disinfectant of commercial eggs to preserve function quality

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ABSTRACT

Ozone is a strong oxidant used for the disinfection of surfaces, drinking water and foods. However, since ozone not only destroys bacteria but may also damage eggs, it is necessary to clarify the effects of ozone treatment on commercial egg components. In this study, different doses of ozone gas, 2, 4, 6 and 10 ppm were used. *Salmonella entritidis* infected eggs were dosed in different experimental groups at different doses of ozone for 15 min. Factors such as TBC, *Salmonella entritidis*, *p*H of egg contents, shell hardness, peroxide content in egg contents and albumen viscosity were measured after 10 days of application. The best amount of ozone gas was obtained at concentrations of 6 and 10 ppm for disinfection of different parts of the egg. While the amount of peroxide produced at 10 ppm was more than standard and harmful. Generally, the application of ozone gas at doses of 4 ppm, especially at 6 ppm, showed the best results for the measured factors. The use of gas at 6 ppm for 15 min can be used to keep the eggs fresh with maximum disinfection. This can be done in the egg industry, in order to remove *Salmonella entritidis* and maintain the quality of commercial eggs.

Keywords: Commercial eggs, Ozone, Peroxide, *Salmonella entritidis*, Viscosity

Disinfection of the egg shell surface is an important tool to prevent egg spoilage and egg-related diseases. The penetration of microorganisms through the shell may result in embryonic mortality, weak chicks, high chick mortality and poor chick growth. Salmonellosis belongs to the most important zoonosis throughout the world (Braun *et al.* 2011). Surveillance performed by the Enter-net National Reference Laboratories since 1993 has shown that *Salmonella entritidis* (*S.e.*) continues to be the predominant *Salmonella entritidis serovar* in Western Europe (Prabha *et al.* 2015). Ozone (O_3) is known as a highly reactive antimicrobial agent. In fact, ozone treatment has been extensively tested for potential application in the food industry, i.e. decontaminating hatcheries, hatching eggs, and poultry carcasses (Perry *et al.* 2011). Because of the spontaneous decomposition into non-toxic-oxygen and readily biodegradable substances there are nearly no residues (Yüceer *et al.* 2016). Fuhrmann *et al.* (2010) showed that even at low ozone concentrations, cuticula proteins of the egg can be destroyed by oxidation of amino acids and three-dimensional structures (Fuhrmann *et al.* 2010). Recent studies of Yousef *et al* (2016) demonstrated that a dose of 12–14% ozone wt/wt (generator) for 10 min. results in a 5.9 log10 reduction.

MATERIALS AND METHODS

Eggs: In this research, egg samples were taken from a specific farm, and laying hens in the farm have the same and distinct dietary rations. The sample was taken from the farm on a given day (after two days of storage in the farm). Five groups of eggs, the same weight, were used as 5 treatments, which were a control group and 4 experimental groups. In the control group, ozone gas was not used, but in experimental groups, ozone gas was used for 15 min at doses of 2, 4, 6, 10 ppm. In each treatment, there were 6 eggs from the Hy-line W36 strain, each experiment being tested for 7 eggs per replicate. The sampling method was completely random and used to apply ozone gas from an ozone gas distributor. The weight of the eggs were 55–65 g and room temperature was 20–25°C. It should be noted that the laying hens were 40 weeks old.

Ozone generation equipment and treatments: Gaseous ozone was produced using a plate type ozone generator (ozone injector 55, Shafa company, Iran) in a custom-made ozone glass vessel with a diffuser as a gaseous ozone injector. Treatments consisted of control (untreated) eggs, and eggs treated with gaseous ozone at concentrations of 2, 4, 6 and 10 ppm, and exposure times of 15 min.

Storage time: Due to the farm experimental study, this research was avoided by laboratory contamination. For this purpose, serological samples were taken from suspected *Salmonella entritidis* infection (RSAT) of commercial hens. Eggs were transferred to the laboratory and were carried out to cultivate and *Salmonella entritidis* isolated from egg shells. In the *Salmonella entritidis* isolate, egg shells were stored for 10 days at 24°C.

Eggs were classified into 2 groups. In the first group for chemical index such as peroxide, viscosity and *p*H, eggs

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were treated by ozone, and samples stored at 24°C for 10 days.

In the second group, for maintenance of TBC and *Salmonella entritidis* infection, storage was carried out for 10 days and then they were ozonized.

TBC (*Total Bacterial Count*): First, in the vicinity of the flame, each egg was broken and separated from the shell, white and yolk were placed in the glass plates and homogenized. Then, weighed and mixed 10 g each of the samples in the salt peptone diluent produced inside the Erlenmeyer. Different dilutions were prepared and taken from dilutions of 2 ml and added to 1 ml sterile petri dishes. The PCA culture was added @ 15 ml per petri dish of potassium agar and the pellets were stirred in 8 shape. Subsequently, the plates were incubated at 30°C in incubator for 72 h. After incubation, the number of colonies per plate were calculated according to the following formula:

$$N=\frac{\Sigma C}{V(n_1+0.1n_2)d}$$

ΣC, total number of colonies counted in petri dishes were of two consecutive randomly selected volumes; V, Volume of fluid inoculated in each petri dish in ml; n_1, number of petri dishes at the first dilution selected; n_2, number of petri dishes in the second selected dilution; and d, Dilution factor for the first dilution selected.

Salmonella entritidis: First, in the vicinity of the flame, each egg was broken and separated from the shell, white and yolk were placed in the glass plates and homogenized. Then 25 g of shells and 25 g of white and 25 g of yolk were weighed into Erlenmeyer containing 225 ml of peptone water buffer. Then, all of the ARLENs were incubated for 18 to 20 h at 37°C. Laminar-flow hood was inoculated from all of the Erlenmeyer on the RVS broth and Muller Kauffman Tetrathionate broth. The Kaufman tube was incubated at 37°C for 24 h and the RVS tube was incinerated at 41.5°C for 24 h. Then, from both tubes, a linear culture is plotted on the XLD medium and the brilliant green agar BGA. Then, each of the 4 plates were incubated at 37° C. In the case of the growth of red colonies with a black or yellow center on the XLD agar culture and suspicious colonies of the same environment, or yellow or brown, on a Gray agar culture, at least 5 colonies were removed with sterile loop and, lineally cultivated on the agar nitrite culture. The agar-containing plate was incubated for 37 h in 24°C. Grown colonies were cultured on TSA agar and lysine dextrocidase broth and tryptone water and urea agar for biochemical confirmation tests.

Urea agar results: The urea culture medium did not change.

Result of lysine decarboxylase: The colour of the culture is purple. And the results are positive if *Salmonella entritidis* is present and in the absence of *Salmonella entritidis,* it is reported negative in 25 g of the sample.

Albumen viscosity: The egg was broken and the resulting albumen was collected in a container and the viscosity (anton-poar, mcr-301) was measured. Albumin measured at 20°C.

p*H measurements:* Egg albumen (3×10 eggs) for each treatment was homogenized for 20 s using a Waring Blender Model 32 BL 80 (Waring, Torrington, CT, USA) and then the *p*H of egg yolk was measured using a *p*H meter (Hanna Instruments, Woonsocket, RI, USA).

Peroxide: First, the egg yolk was poured into an Erlenmeyer, then some solvent (normal hexane or petroleum ether or diethyl ether) was applied to the yolk in the Erlenmeyer and it was allowed to stand for 24 h until the egg oil extracted solvent. After oil separation and evaporation of the solvent, some of the oil was weighed and 3:2 acetic acid-chloroform added. Next, 0.5 ml of potassium iodide was added to the solution and laid for 1 min in the dark. By adding 30 ml of distilled water and a few drops of reagent starch, changing the colour to violet, the presence of peroxide in the solution was proven and titrated with sodium thiosulphate 0.01 N to purify the violet colour and obtain the amount of thiosulphate consumed. Peroxide (mg/kg) was calculated from the following equation:

$$P = \frac{N \times V \times 1000}{m}$$

where, N, thiosulphate normal; V, volume of thiosulfate consumption; m, sample weight.

If no change in purple colour was achieved, then peroxide was considered to be zero.

Statistical analysis: Using a CRD design, a completely randomized design was used to compare the mean with PLSD at the probability level of 0.05 with SAS software version 9.3, 2013.

RESULTS AND DISCUSSION

TBC of Albumen: Table 1 shows the changes in TBC of albumen values of untreated and ozone-treated eggs during storage for 10 days at 24°C. Statistical analysis showed that the interactions of three factors (Week × Concentration × Time) were significant ($P<0.05$) and by increasing the concentration of ozone, the TBC of albumen values has decreasing trend and at concentration ozone of 10 ppm, reaching the lowest TBC. According to Table 1, by increasing the amount of ozone concentrations, TBC decreases in albumen, with the lowest TBC observed at 10 ppm. Also, the reduction in TBC levels at 6 and 10 ppm is significant in comparison to 2 and 4 ppm, but does not differ significantly from 6 ppm and 10 ppm. In our study, data of control group as compared with other experimental groups were significant ($P< 0.05$) (Table 2).

TBC of yolk: Statistical analysis for TBC of yolk showed in Table 1 that the interactions of three factors (Week × Concentration × Time) were significant ($P< 0.05$). From Table 1 it could be seen that by increasing the concentration of ozone, the TBC of yolk values has decreasing trend and at concentration ozone of 10 ppm, reaching the lowest TBC. By increasing ozone concentration, the amount of TBC in

Table 1. Effect of the ozone treatments on eggs during 10 days of storage time

Parameter	2 ppm	4 ppm	6 ppm	10 ppm	SE	P-value
TBC (albumen) [Cfu(25/gr)]	1650.00[a]	156.67[b]	18.17[c]	12.33[c]	38.33	0.00
TBC (yolk) [Cfu (25/gr)]	180.00[a]	17.33[b]	14.67[b]	11.50[b]	3.18	0.00
TBC (shell) [Cfu(25/gr)]	493.33[a]	316.67[b]	266.67[c]	175.00[c]	10.86	0.00
SE (shell)(Cfu/gr)	158.33[a]	15.83[b]	0.00[c]	0.00[c]	4.75	0.00
SE (contents)(Cfu/gr)	176.67[a]	43.16[b]	0.00[c]	0.00[c]	5.17	0.00
*p*H (albumen)	9.20	9.29	9.23	9.19	0.04	0.50
*p*H (yolk)	6.26	6.15	6.32	6.24	0.12	0.79
Viscosity (mPa/s)	137.67	135.47	145.50	122.70	12.48	0.65
Peroxide (mEq/kg)	0.13[b]	0.67[b]	0.76[ab]	1.18[a]	0.22	0.02

Ozone concentration (ppm). Values are means±SE. Different letters in same rows were significantly differences (P<0.05).

Table 2. Comparison of control (ozone free) with ozone treatments on eggs

Parameter	Mean square	P value
TBC (albumen) [Cfu(25/gr)]	2.66×10^9	0.00
TBC (yolk) [Cfu (25/gr)]	1.49×10^{10}	0.00
TBC (shell) [Cfu (25/gr)]	2.87×10^{11}	0.00
SE (shell) (Cfu/gr)	1.22×10^9	0.00
SE (contents) (Cfu/gr)	1.78×10^9	0.00
*p*H (albumen)	0.03	0.10
*p*H (yolk)	0.06	0.22
Viscosity (mPa/s)	2666.67	0.02
Peroxide (mEq/kg)	0.51	0.18

Values are means±SE. Different letters in same rows were significantly differences (P<0.05).

yolk egg decreases. It is noteworthy that TBC in ozone concentrations is significantly reduced by more than 2 ppm (4, 6, 10 ppm) compared to 2 ppm, but there is no significant difference in TBC values at doses of 4, 6 and 10 ppm. According to Table 2, data of control group as compared with other experimental groups were significant (P< 0.05).

TBC of egg shell: Statistical analysis in Table 1 for TBC of egg shell showed that the parameter was effective from ozone and by increasing the concentration of ozone, the TBC of egg shell values has decreased and the lowest TBC was observed at concentration ozone of 10 ppm (P< 0.05). The egg shell TBC has been reduced by increasing ozone concentrations and there is no significant difference between the different concentrations of ozone. According to table 2, data of control group as compared with other experimental groups were significant (P< 0.05).

Salmonella entritidis *on the Egg shell:* Table 1 shows *Salmonella entritidis* on the egg shell values of ozone-treated eggs during 10 days of storage and in ozone-treated groups, *Salmonella entritidis* on the shell decreased by increasing ozone dose (P< 0.05). By increasing the amount of ozone, the amount of S.e. in the egg shell decreases so that at 6 and 10 ppm, the S.e. value reaches zero. With accuracy in Table 2, the *Salmonella entritidis* on the shell of control group as compared with other experimental groups were significant (P< 0.05).

Salmonella entritidis *(internal of egg):* Table 1 shows *Salmonella entritidis* in the internal values of untreated and ozone-treated eggs during 10 days of storage. The *Salmonella entritidis* in the internal of the ozone-treated eggs decreased by increasing ozone dose, so in 6 and 10 ppm *Salmonella entritidis* in the internal egg were zero (P> 0.05). The egg shell internal of egg decreases sharply with the increase in ozone and reaches zero at 6 and 10 ppm. With accuracy in Table 2, the *Salmonella entritidis* on the internal of control group as compared with other experimental groups were significant (P< 0.05).

Albumen viscosity: Viscosity of the albumen affects the functional properties of eggs during whipping and emulsifying (Kannan *et al.* 2013). Thick albumen progressively liquefies and thins with time, transforming itself into thin albumen due to the changes in the complex lysozyme–ovomucin. Any adverse effects on the viscosity of albumen affect these properties and would make eggs unsuitable for industrial use (Spada *et al*. 2012). The statistical analyses showed that interactions of (Week × Concentration × Time) were important. According to Table 1, the albumen viscosity in ozone treatment is not statistically significant (P>0.05). According to Table 1, increasing or decreasing the amount of ozone concentration does not have effect on viscosity. In fact, the same process of exposure to viscosity is not observed. That is, about 6 ppm is slightly different from the others (2,4 and 10 ppm). It is observed that in 6 ppm, viscosity increased to 2, 4 and even 10 ppm, and the amount of viscosity increased in this ozone concentration (6 ppm). In Table 2, comparison of control group with other groups were significant (P< 0.05).

p*H measurements:* Freshly laid eggs have an albumen *p*H that lies between 7.6 and 8.5, and contain 1.44 –2.05 mg CO_2/g of albumen (Perry *et al*. 2011, Yüceer *et al.* 2016). In our study, the *p*H of albumen was around 9 and *p*H of yolk was 6. In fact, *p*H was not affected from ozone. According to table 2, the difference between the control and ozone treatment groups for *p*H of albumen and yolk were not statistically significant (P>0.05).

Peroxide: Statistical analysis showed that interactions of factors (Week × Concentration × Time) were not significant between control and experimental groups (Table 2) (P>0.05). However, the effects of ozone concentration between experimental groups were

statistically significant (Table 1). The increasing of ozone concentration had a significant effect on the amount of peroxide. It was observed that by increasing the amount of ozone from 2 to 10 ppm, the amount of peroxide increases. So that up to 6 ppm, egg peroxide value is not significantly different in 2 and especially 4 ppm, but by increasing the dose of ozone to 10 ppm, the amount of peroxide is significantly more than 6 ppm.

Many human pathogens grow predominantly as biofilms rather than in planktonic mode (Giaouris *et al*. 2013). Bacterial biofilms are broadly described as a microbial derived sessile community characterized by cells that are attached to a substratum or to each other and are embedded in a matrix of extracellular polymeric substances (EPS), and exhibit an altered phenotype with respect to growth rate and gene transcription (Giaouris *et al.* 2013, Goo-Hee and Kyung-Haeng, 2012). Formation of bacterial biofilms on food contact surfaces, on food processing equipment and in potable water distribution systems contributes to food spoilage, cross contamination of food products and spread of food borne pathogens (Kim and Wei 2012) and therefore represent a major challenge in food industry (Borges *et al.* 2013). Moreover, biofilms are more resistant to various environmental stresses and the actions of applied antimicrobial treatment.

Numerous ozone applications have been installed throughout the food industry in the worlds. The benefits to public food safety are major, especially related to the food hazards identified in the President's Food Safety Initiative. The presence of bacterial contamination at the egg level and its internal contents can have several factors (Goo-Hee and Kyung-Haeng, 2012). The contamination of the laying hens and the transmission of ovaries or fecal contamination on egg shells and the lack of appropriate collection and manipulation of poultry workers can be of great importance in increasing these infections. On the other hand, the presence of *Salmonella entritidis* bacteria, especially *Salmonella entritidis*, can play a very important role in human food contamination through eggs. TBC and *Salmonella entritidis* have been considered for eggs for human consumption (for a S.e. of zero and for TBC values were less than 10 CFU). The results obtained in this study show the very good effect of ozone gas in the applied doses, especially at concentrations of 6 and 10 ppm, which minimizes the contamination. According to studies, ozone gas can not only affect the surface of the egg, but also penetrate the holes on the egg shell to play an important role in disinfecting the contents of eggs, including yolk and albumen. The degree of ozone penetration across egg shells was not correlated with gas concentration in the environment. This is in agreement with previous reports indicating that there are a high number of pores on the egg shell blunt end just above the air cell (Fry *et al.* 2015).

Braun *et al* (2011), have investigated the feasibility of gaseous ozone to reduce the number of microorganisms on the shell surface, of *Salmonella enteritidis* (S.e) in particular, of avian hatching eggs. In their research, shell eggs were externally contaminated with S.e. to contain either 102–104 or 105–106 cfu/shell. Subsequently, the eggs were exposed to several ozone concentrations ranging from 0.5% to 5% wt/wt in combination with two relative humidity at room temperature. Results of Braun *et al.* 2011), were displayed a complete inactivation of S.e. on egg shell by using an ozone concentration of 1% (wt/wt) for 120 min. Considering higher concentrations of S.e. on the shell ozone treatment caused approximately a 6 log10 reduction (Braun *et al.* 2011).

In our study, albumen *p*H increased, in control than ozone-treated eggs (Table 1), but the application of ozone seemed to prevent drastic changes in albumen *p*H ($P>0.05$). The albumen *p*H values in ozone-treated eggs were higher than in control eggs during ozone treatments. Although yolk *p*H increased during ozonation, the increase of ozone concentrations induces to invariable *p*H in all eggs. In fact, ozone has no effect on yolk pH. In control and ozone-treated eggs, yolk *p*H was around 6. The *p*H of yolk in freshly laid eggs is generally about 6.0 and gradually increases to 6.4–6.5 during storage.

The viscosity of the albumen affects the functional properties of eggs during whipping and emulsifying (Kannan *et al*. 2013). Thick albumen progressively liquefies and thins with time, transforming itself into thin albumen due to the changes in the complex lysozyme–ovomucin. Any adverse effects on the viscosity of albumen affect these properties and would make eggs unsuitable for industrial use (Kannan *et al.* 2013, Kemps *et al*. 2010, Spada *et al.* 2012). Albumen viscosity may be used as a tool for assessing egg quality, in that the gelatinous structure of thick albumen changes physical and chemical characteristics and gradually breaks down into a clear liquid losing its consistency during storage. Albumen viscosity is also a potential tool for the assessment of egg quality. The viscosity of albumen values reported by others varied between 10 and a few hundred mPa/s (Kannan *et al.* 2013). Variation between measurements is perhaps caused by discrepancies in sample preparation and measurement protocol. Albumen, a pseudo plastic fluid, is a thixotropic material (Ruth *et al.* 2013) and its viscosity depends on the shear force. The viscosity of the albumen decreased during storage (Table 1) confirming earlier results obtained by Kannan *et al.* (2003), and Kemps *et al.* (2010).

In our study, for treated eggs, albumen viscosity values increased with ozone concentration and in 6 ppm it is higher than other ozone concentrations. The albumen viscosity depends on the ovomucin–lysozyme complex (Spada *et al*. 2012). When lysozyme is present in the complex, it becomes stronger and its destabilization changes due to *p*H increase during storage (Kemps *et al*. 2010, Ruth *et al*. 2013, Spada *et al.* 2012). The liquefaction of albumen occurs because of the increasing *p*H. It is influenced by the ovomucin–lysozyme complex, which results in changes in viscosity of the albumen during storage (Kemps *et al.* 2010, Ruth *et al.* 2013). It is possible that the ozone treatments minimize changes in carbohydrate and protein moieties involved in

formation of ovomucin complex, resulting in a loss of gel-like structure during storage and minimize changes in *p*H and maintained albumen quality (Hernandez-Ledesma and Chia-Chien 2013). So, the results indicated the effect of ozone treatment is not significant in comparison with the control group (P>0.05).

Investigations of the fatty acid composition of egg yolk after ozone exposure are also lacking in the literature. To understand the mode of action of ozone against organic molecules, experiments with isolated lipids were carried out in the past. It was assessed earlier that ozone has different affinities to individual fatty acids. In the Table 1, ozone has little impact on the oxidation of fatty acids of egg yolk, in fact by increasing the ozone concentration, the amount of peroxide is reduced, but it is noteworthy that peroxide is the primary product of fatty acids oxidation, high peroxide value due to food corruption, therefore, the decreasing peroxide value during the ozone treatment in this study indicates that there is no egg corruption that it is be done better than 6 ppm.

Our study suggests that, ozone treatments helped to maintain egg quality for a longer time. Considering the effect of ozone gas on the factors measured in this study, it is observed that ozone in the dose of 4, 6 ppm yields the best results. According to the results, 10 ppm has the greatest impact on the studied parameters, but reduced the nutritional quality of the egg and increased parameters such as peroxide, which is not desirable. So, 6 ppm improved the measurement of factors and maintains the quality of the egg's nutrition to a long time. Thus, ozone treatment of eating eggs as one of the most effective methods of disinfection can be used in the food industry.

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