



## A COMPARISON BETWEEN OZONATION AND THERMAL PROCESS IN RELATION TO COW'S MILK ATTRIBUTES WITH EMPHASIS ON PATHOGENS

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### ABSTRACT

Effect of different ozonation dozes on some physicochemical and microbiological properties in raw and pathogens inoculated milk samples in comparison with conventional heat treatment with emphasis on pathogens was aimed to be investigated.

Raw cow' milk was divided into eight equal portions, 1<sup>st</sup> portion was heat treated at 72°C for 15 sec then rapidly chilled to room temperature. Other 7 portions were treated with ascending ozonation times, namely, nil (served as a control), 5, 10 15, 20, 25 or 30 min using an ozone generating device from the air at the rate of 400 mg O<sub>3</sub>/h. In another experiment, five portions of previously autoclaved skimmed milk (at 115°C /15 min) was separately inoculated with 0.1% of 24-h age broth cultured with *Staphylococcus aureus* ATCC25923, *Bacillus cerues* ATCC10876, *E. coli* ATCC25922, *S. typhimurium* ATCC 14028 or *Sh. flexneri* ATCC 12022). All inoculated milks were ozonized for nil, 5, 10 15, 20, 25 or 30 min at the same rate and immediately tested for the survival count of the studied pathogenic strains.

The obtained data indicated that, both raw milk treatments studied, namely thermal and ozonation (for more than 5 min) lowered significantly counts of total bacteria, yeasts & molds, *Enterobacteriaceae* and psychrotrophic bacteria than those in raw cow' milk. All counts of pathogenic bacteria were less than one cfu/ml after 30 min ozonation., *E.coli* and *S .typhimurium* counts were less than one after only 20 min ozonation. No significant differences were detected in all physicochemical properties among all milks treated whether thermally or by ozonation.

As a conclusion, ozonation treatment of raw and pathogenic inoculated milk up to 20 min at the rate of 400mg O<sub>3</sub> /h had significantly improved the microbiological quality being able to cause decimal reductions in the milk native flora. Although there has been a marked decrease in the studied pathogenic bacteria as a result of ozonation, this is not enough for assurances, indicating the need for thermal treatment to assure the complete elimination of pathogenic microbes. Additionally, no significant changes were recorded in the physicochemical properties of the ozone treated milk up to 30 min at the same rate. Thus, these findings feature the bubbles of ozonation as a substitute to diminish the load of microbes in the raw milk, improve quality and increase the milk shelf-life before different processing.

**Keywords:** Gram positive and negative pathogens, *Enterobacteriaceae*, Psychrotrophic bacteria

### INTRODUCTION

Conventionally, heating is the common procedure applied to control the load of microbes in milk. It is very straightforward and efficient, but may lead to several sensory acceptability and nutritional losses in the resultant product, particularly when heat treatment is implemented in poor quality milk, which possesses a high load of microbes and hence more heating process (temperature degree and/or time) is needed. A substitute method to decrease the load of microbial load of the milk is the use of substances that deactivate microorganisms with no change in milk safety and quality (e.g. peroxide, ozone, and CO<sub>2</sub>). The use of these mate-

rials may reduce the time and temperature required in the pasteurization of milk due the reduced initial counts of microorganisms in milk. Above all, these materials may setback the production of enzymes by microorganisms during storage before heating treatment and improve the stability of protein in milk during its shelf-life (**Pedras et al 2012**).

On the other hand, innovative, easy to implement, and rapid technologies are required for the modern food industry to meet the never ending demands of consumers (e.g. fresh and safe ready-to-eat products). Ozone was found to be a powerful tool for sanitation and gaining growing interest. The sanitation with ozone may, then, meet the standards of modern food industry. It also may accomplish the regulations of different food safety organizations as well as the consumer acceptability (**Kim et al 1999**). However, there are over than 3000 installations of waste treatment using ozone across the world (**Rice et al 2000**). Speaking from the viewpoint of health, ozonation gained popularity in several countries (e.g. New Zealand, Japan, Australia, North America, and several European countries), in varying degrees (**Tiwari and Rice, 2012**).

Ozonation has several merits that make it a technology to be considered in the food industry; it is the most powerful sanitizer; it decomposes rapidly to oxygen leaving no residues. Low concentrations of ozone are quite enough to be effective (**Khadre et al 2001**). It acts strongly against extended spectrum of microorganisms, i.e. bacteria, fungi, viruses, protozoa, and bacterial as well as fungal spores (**Roushdy et al 2011**).

The relative sensitivity of bacteria to ozone is not widely agreed upon and the results in this concern are not inclusive. This may be attributed to different factors like the strain of cultured microorganisms and other related factors including age and density of cultures and the presence of culture media components that require ozone. Also amongst other factors are the method of ozone application (gas bubbles or uniform aqueous solution), the used device and the accuracy of measuring the microbial potency (e.g. single point determinations vs systematic kinetics). The mechanism by which ozone deactivate microorganisms is complicated. That is because ozone attacks several constituents of cells (e.g. unsaturated lipids, proteins, nucleic acids in the cytoplasm, respiratory enzymes in cell membranes, and coat components of spores, e.g. peptidoglycans in cell envelopes,

enzymes and, and proteins and peptidoglycan (**Khadre et al 2001**).

Recently, ozone industries has been developing the concept of continuous disinfection aiming to keep any imported contamination under control at all times hence minimizing its spread to the final product to guarantee the improving overall product quality and customer satisfaction (**Brodowska et al 2018**).

Ozonation diminished the initial load of different microorganisms (e.g. total count of aerobic mesophiles, yeasts, molds and lactic acid bacteria) by approximately 2 log cycles. Ozonation has no effect on the physicochemical properties of Minas Frescal cheese samples (**Cavalcante et al 2013**). Therefore, this research aims were assess the effect of different ozone dozes on some chemical and microbiological properties of raw and pathogens inoculated milk samples compared with that heat treated.

## MATERIALS AND METHODS

### 1. Materials

Fresh full cream cow's milk and skimmed milk were obtained from the herd of the dairy cattle at Faculty of Agriculture, Cairo University. Five food borne pathogens were used, including tow Gram positive bacteria, namely *Staphylococcus aureus* ATCC25923 and *Bacillus cerues* ATCC10876 and three Gram negative bacteria, namely *Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC 14028 and *Shigella flexneri* ATCC 12022. These strains were obtained from Egyptian Microbiology Culture Collection (EMCC) at Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

### 2. Experimental procedure

#### 2.1. Designing of milk ozonation

Forty kilograms of raw cow' milks were divided into eight equal portions, 1<sup>st</sup> portion was heat treated at 72°C for 15 sec then rapidly chilled to room temperature (25°C). Other 7 portions were treated with ascending ozonation times, namely, nil (served as a control), 5, 10 15, 20, 25 or 30 min using an ozone generating device from the air (Electric Anion Ozonizer, Healthy Life, Model, AK-102, UK) at the rate of 400 mg O<sub>3</sub>/h.

## 2.2. Survival screening of some pathogenic strains in milk towards different ozonation doses

Twenty five kilograms of fresh skim milk were autoclaved at 115°C /15 min then chilled to room temperature. Thereafter, the sterilized milk was divided into equal five portions. Two portions were separately inoculated with 0.1% of 24-h age broth cultured with Gram positive pathogenic bacterial strain of *Staph. aureus* or *B. cereus*. Other three portions were individually inoculated with 0.1% of 24-h age broth cultured with Gram negative bacterial strain of *E. coli*, *S. typhimurium* or *Sh. flexneri*. All inoculated milk samples were ozonized for nil, 5, 10, 15, 20, 25 or 30 min at the rate of 400 mg O<sub>3</sub>/h and immediately tested for the survival count of studied pathogenic strain.

## 3. Analytical methods

### 3.1. physio-chemical analyses

Dry matter (DM), protein and ash contents were determined according to **AOAC (2012)**. The fat and titratable acidity (TA) contents were determined according to **Ling (1963)**. Lactose content was determined according to the method reported by **Lawrence (1968)**. The milk pH value was measured electrometrically using a digital pH meter (Hanna model 8417) at 20 °C after calibrating with fresh pH 4.0 and 7.0 standard buffers according to the methods of **BSI (1989)**.

### 3.2. Microbiological examination

The total bacterial count (TBC) was enumerated according to **Houghtby et al (1993)** using Plate Count Agar at 32°C for 48h. *Enterobacteriaceae* were determined according to **Gaya et al (1987)** using Violet Red Bile Glucose Agar (VRBGA) at 37°C for 24h. Psychrotrophic bacteria were enumerated according to **Marshall, (1992)** using Plate Count Agar at 7°C for 10 days. Yeasts and Molds were determined using Malt Extract Agar at 25-27°C for 4 days as suggested by **Harrigan and McCance (1966)**. *Salmonella* sp. was enumerated according to standardized procedures for bacteriological examination of food products as described by **AOAC (2012)** using Xylose Lysine Deoxycholate Agar for 24 ± 3h at 37°C. Baird Parker Agar was used for determined staphylococci as in **Oxoid Manual (1982)**. The plates were incubated at 37°C for 48 h. *E.coli* was enumerated according

to the method described in **Nunez et al (1985)** using Violet Red Bile Lactose Agar incubated at 44°C for 24 h. *B. cereus* was enumerated according to **FDA (2012)** on Mannitol Yolk Polymyxin (MYP) Agar fully absorbed before incubating for 24 h at 30°C. *Shiegilla* sp. was enumerated according to standardized procedures for bacteriological examination of food products as described by **AOAC (2012)** using Xylose Lysine Deoxycholate Agar for 24 ± 3h at 37 °C.

### 3.3. Statistical analysis

Statistical analysis was performed according to **SAS (1990)** using General Linear Model (GLM) with main effect of treatments. Duncan's multiple range was used to separate among of three replicates at P ≤ 0.05.

## RESULTS AND DISCUSSION

### 1- Microbiological quality of cow's milk in relation to ozonation versus conventional heat treatment

Data given in **Table (1)** indicated that, both of heat and ozonation treatments led significantly to lower both of total bacterial and yeasts & molds counts of milk. Milk samples ozonized at the rate of 400mg O<sub>3</sub>/h for 5 or 10 min contained total bacterial and yeasts & molds counts higher than those of heat treated one. Non-significant differences were observed either in count of total bacterial or yeasts and molds between heat treated milk sample and those ozonized for 15 min (**Table, 1**).

The reduction in cell count of milk as affected by heat treatment may be due to denaturing the enzymes in the bacteria, damaging the bacterium's cell envelope. While, **Todar (2012)** explained that, ozonation may be lead to proteins and fatty acids of the envelope lose their shape leading to their weakening. **Burley (1985)** reported that the reduction in cell count in ozone treated cow milk may be due to that the bodies of bacteria are tightly closed by a comparatively solid-cell membrane where the vital processes are restrained through a system of complex enzymes.

Ozone intrudes the metabolism of bacterium-cells, clearly through inhibiting and blocking the action of the enzymatic control system. An appreciated amount of ozone intrudes the cell membrane, leading to the eradication of the bacteria. Similar findings were reported by **Metwally et al (2011)**.

Surviving of *Enterobacteriaceae* determined by enumeration and listed in **Table (1)** indicated that, heat treatment (72°C for 15 sec) or ozonation of milk (for 15,20,25 or 30 min at the rate of 400mg O<sub>3</sub>/h) caused reductions in *Enterobacteriaceae* count from 2.18 log cfu/ml in raw milk to less than one log cfu/ml in heated milk. While, milk ozonation only for 5 or 10 min resulted in reduction of *Enterobacteriaceae* count from 2.18 to 2.00 or 1.50 log cfu/ml respectively. The reduction in *Enterobacteriaceae* in ozone treated milk may be due to that oxidation and, hence, the destruction of the cell wall and cytoplasmic membrane by ozone. Primarily, ozone assails glycoproteins and glycolipids of the bacterial cellular membrane, as well as certain amino acids, such as tryptophan. Further, **Dosti et al (2005)** added also that, ozone may react with the sulfhydryl groups of some enzymes, which lead to impaired cellular activity. Therefore, the resulting shifts in membrane permeability cause cell lysis leading to improvements in quality of milk and increments in shelf life till heating process. Similar facts were recommended by **Cavalcante et al (2013)** and **El-Loly et al (2013)**.

Regarding Psychrotrophes counts, the results in **Table (1)** revealed that, no significant differences existed in Psychrotrophes counts between heat treated and ozone treated milk samples for 20 min. Therefore, it could reported that, ozonation of raw milk at the rate of 400mg O<sub>3</sub>/h for 15 min led to gain a microbiological quality similar to that of heat treated milk at (72°C for 15 sec). These results are in line with those found by **Cavalcante et al (2013)** who observed that ozone gas bubbling might be adopted as a milk pre-process aiming at a decrease in microbial count of raw milk; however, this treatment did not lead to dispose of the required milk thermal process. **Selma et al (2008)** stated also that, gaseous ozone (10000 ppm for 30 min) treatment was effective on blanched cantaloupes to diminish mesophilic, psychrotrophic and yeasts & molds loads. Milk with ozone treatment at the rate of 400mg O<sub>3</sub>/h for 5 min had no influence on microbiological quality of raw milk. These results are in coincidence with those of **Cavalcante et al (2013)** who observed that the ozonation for 5 min was not capable of reducing the counts of milk microbes.

**Table 1.** Microbiological situation (log cfu/ml) of cow's milk as affected by alternative conventional heat or ozonation at the rate of 400 mg O<sub>3</sub>/h for different times

Microbiological count (log cfu/ml)	Raw	Treatment of cow's milk						
		Heat at (72°C/15sec)	Ozonation time (min)					
			5	10	15	20	25	30
Total bacteria	7.22 <sup>a</sup>	4.32 <sup>d</sup>	6.15 <sup>b</sup>	5.27 <sup>c</sup>	4.12 <sup>d</sup>	3.45 <sup>e</sup>	3 <sup>e</sup>	2.40 <sup>f</sup>
Yeasts & Molds	3.11 <sup>a</sup>	1.05 <sup>d</sup>	2.9 <sup>b</sup>	2.45 <sup>c</sup>	1.11 <sup>d</sup>	1 <sup>d</sup>	0.9 <sup>d</sup>	0.5 <sup>e</sup>
Enterobacteriaceae	2.18	<1	2.00	1.50	<1	<1	<1	<1
Psychrotrophes	3.15 <sup>a</sup>	1.60 <sup>e</sup>	2.9 <sup>ab</sup>	2.71 <sup>b</sup>	2.50 <sup>c</sup>	2.01 <sup>d</sup>	1.8 <sup>e</sup>	1.17 <sup>f</sup>

cfu/ml:colony forming unit/millimeter

Means with the same letter at any position did not significantly differ (p>0.05).

## 2- Screening of different ozonation doses on survival of some pathogenic strains in milk

Survival of some pathogens (log cfu/ml) inoculated in sterilized skim milk as affected by the ozonation time (min) at the rate of 400mg O<sub>3</sub> /h are present in **Table (2)**. The data stated that, all counts of pathogenic bacteria were <1 cfu/ml after 30 min ozone treatment. The mechanism by which ozone deactivate microorganisms is complicated. That is because ozone attacks several constituents

of cells (e.g. unsaturated lipids, proteins, nucleic acids in the cytoplasm, respiratory enzymes in cell membranes, and coat components of spores (e.g. peptidoglycans in cell envelopes, enzymes and, and proteins and peptidoglycan).In details, *E. coli* and *S. typhimurium* counts were less than one after 20 min ozone treatment. Although there has been a marked decrease in the studied pathogenic bacteria as a result of ozonation, this is not enough for assurances, indicating the need for thermal treatment at temperature degrees over the range

needed for the growth of normal cells leading to progressive loss of bacterial viability to assure the complete eradication of pathogenic microbes and hence food safety. Similar observations were reported by Proudlove (1989), Kim et al (1999), Selma et al (2001), Aydogan and Gurol (2006), Patil et al (2011), Prabakaran et al (2012), Fontes et al (2012), Cavalcante et al (2013), Hye-Jung et al (2014) and Shreya et al (2015).

**3- Effect of ozonation on chemical properties of cow's milk**

The statistical analyses revealed that, neither the kind of treatment (whether, thermal or ozonation) nor the ozonation doze led to any significant

difference among all physiochemical properties studied. Whereas, the results of Table (3) showed total solids content of various milk samples ranging between 12.55% (raw milk) and 12.64%. While, fat content showed levels ranging from 3.1 to 3.2%, whilst protein content (TNx6.38) showed variation ranging from 3.2 to 3.4%. Lactose content showed variation ranging from 4.45 to 4.53%, while ash content showed levels ranging from 0.76 to 0.77%. Treatable acidity % showed variation ranging from 0.17 to 0.18%, while pH value showed variation ranging from 6.64 to 6.70.

These findings are in coincidence with those of Cavalcante et al (2013), who declared that the ozonation up to 15 min did not change the physicochemical criteria in raw milk.

**Table 2.** Survival of some pathogens (log cfu/ml) inoculated in sterilized skim milk as affected by the ozonation time (min) at the rate of 400mg O<sub>3</sub> /h

Ozonation time (min)	Pathogenic strain count (log cfu/ml)				
	<i>E. coli</i>	<i>Staph. aureus</i>	<i>Salmonella typhimurium</i>	<i>B. Cerues</i>	<i>Shigella flexneri</i>
Initial	8.30	8.50	8.40	8.20	7.40
5	5.20	6.08	5.50	7.30	6.80
10	4.70	5.30	4.40	6.60	5.18
15	3.20	3.40	3.30	5.40	4.28
20	<1	0.90	<1	2.70	1.36
25	<1	<1	<1	<1	0.90
30	<1	<1	<1	<1	<1

cfu/ml: colony forming unit/millimeter

**Table 3.** Physicochemical properties of cow's milk as affected by conventional heat or ozonation treatment (at the rate of 400mg O<sub>3</sub>/h for different times)

Property	Raw	Treatment of cow's milk					
		Heat at (72°C/15sec)	Ozonation time (min)				
			10	15	20	25	30
Total solids (%)	12.55 <sup>a</sup>	12.64 <sup>a</sup>	12.65 <sup>a</sup>	12.70 <sup>a</sup>	12.58 <sup>a</sup>	12.60 <sup>a</sup>	12.65 <sup>a</sup>
Fat (%)	3.20 <sup>a</sup>	3.20 <sup>a</sup>	3.10 <sup>a</sup>	3.20 <sup>a</sup>	3.10 <sup>a</sup>	3.20 <sup>a</sup>	3.10 <sup>a</sup>
Protein (%) (TNx6.38)	3.30 <sup>a</sup>	3.40 <sup>a</sup>	3.20 <sup>a</sup>	3.20 <sup>a</sup>	3.30 <sup>a</sup>	3.20 <sup>a</sup>	3.40 <sup>a</sup>
Lactose (%)	4.53 <sup>a</sup>	4.51 <sup>a</sup>	4.50 <sup>a</sup>	4.49 <sup>a</sup>	4.45 <sup>a</sup>	4.46 <sup>a</sup>	4.49 <sup>a</sup>
Ash (%)	0.76 <sup>a</sup>	0.77 <sup>a</sup>	0.77 <sup>a</sup>	0.77 <sup>a</sup>	0.77 <sup>a</sup>	0.77 <sup>a</sup>	0.77 <sup>a</sup>
Treatable acidity (%)	0.17 <sup>a</sup>	0.17 <sup>a</sup>	0.17 <sup>a</sup>	0.17 <sup>a</sup>	0.17 <sup>a</sup>	0.18 <sup>a</sup>	0.18 <sup>a</sup>
pH value	6.64 <sup>a</sup>	6.64 <sup>a</sup>	6.66 <sup>a</sup>	6.65 <sup>a</sup>	6.66 <sup>a</sup>	6.68 <sup>a</sup>	6.70 <sup>a</sup>

TN: Total nitrogen

Means with the same letter at any position did not significantly differ (p>0.05).

### CONCLUSION

Ozonation treatment of raw and pathogenic inoculated milk up to 20 min at the rate of 400 mg O<sub>3</sub>/h had a significantly improving effect on the microbiological quality being able to cause decimal reductions in the milk native flora. Although there has been a marked decrease in the studied pathogenic bacteria as a result of ozonation, this is not enough for food safety assurances, indicating the need for thermal treatment at temperature degrees over the range needed for the growth of normal cells resulting in continuous loss of the ability of bacterial viability to ensure the complete elimination of pathogenic microbes. Additionally, no significant changes were recorded in the physicochemical properties of the ozone treated milk for 30 min at the same rate. Thus, these findings marks the bubbles of ozone as an substitute to diminish of the load of microbes in raw milk, improve quality and increase the milk shelf-life before different processing.

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## مقارنة بين المعاملة بالأوزون والمعاملة الحرارية وعلاقتها بخواص اللبن البقري مع التركيز على البكتيريا الممرضة

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حراريا على أعداد أقل معنويا سواء العد الكلي للبكتيريا، أعداد الخمائر والفطريات، أعداد *Enterobacteriaceae* وأعداد البكتيريا المحبة للبرودة مقارنة باللبن البقري الخام. ولقد كانت أعداد جميع البكتيريا الممرضة في اللبن المعقم الملقح أقل من مستعمرة واحدة لكل مل (cfu/ml) بعد 30 دقيقة من المعاملة بالأوزون. بينما كانت أعداد أي من *E. coli* أو *S. typhimurium* أقل من مستعمرة واحدة لكل مل لبن بعد 20 دقيقة فقط من المعاملة بالأوزون. ولم يكن هناك أي إختلافات معنوية في الخواص الفيزيوكيميائية في كل معاملات اللبن سواء المعامل بالأوزون أو المعامل حراريا.

أخيرا يمكن استنتاج أن معاملة اللبن الخام أو اللبن الملقح بالبكتيريا الممرضة بالأوزون بمعدل 400 مليجرام  $O_3$  كل ساعة لمدة حتى 20 دقيقة قد حسن معنويا من الجودة الميكروبيولوجية وأدى لخفض الحمل الميكروبي في اللبن. ولقد أكدت النتائج أن هذا لا يكون كافيا للتأكد من سلامه الغذاء مما يدل على الحاجة للمعاملة الحرارية اعلى من درجة الحرارة الملائمة لنمو الخلية لإحداث فقد تدريجي لمقدرة الخلية البكتيرية وذلك لضمان القضاء التام على البكتيريا الممرضة وبالتالي سلامة الأغذية. ولم يكن هناك أي تغيرات معنوية ملحوظة بالخواص الفيزيوكيميائية للبن المعامل بالأوزون حتى 30 دقيقة.

الكلمات الدالة: الميكروبات المرضيه الموجبه والسالبه لجرام، *Enterobacteriaceae*، البكتيريا المحبه للبروده

### الموجز

استهدف البحث دراسة تأثير إستخدام الأوزون على بعض الخواص الفيزيوكيميائية والميكروبيولوجية لعينات من لبن خام ولبن ملقح ببكتيريا مرضية بالمقارنة باللبن المعامل بالمعاملة الحرارية التقليدية مع التركيز على البكتيريا الممرضة. حيث تم تقسيم اللبن الخام لثمان أجزاء متساوية الجزء الأول تم معاملته حراريا على 72°م لمدة 15 ثانية ثم تم تبريده لدرجة حرارة الغرفة. أما عينات اللبن الأخرى فتم تعريضها للأوزن لفترات زمنية متزايدة وهي على الترتيب صفر (المقارنة)، 5، 10، 15، 20 أو 30 دقيقة باستخدام جهاز توليد أوزون من الهواء الجوى بمعدل 400 مليجرام  $O_3$  / ساعة Electric Anion Ozonizer (Healthy Life, Model, AK-102, UK).

وفى تجربة أخرى تم تقليج خمس أجزاء من لبن فرز سبق تعقيمه بالايوتوكلاف على درجة 115°م لمدة 15 دقيقة ثم بواسطة 1% من مزرعة سائلة عمرها 24 ساعة لأحد السلالات الممرضة:

*Staphylococcus aureus*, *Bacillus cerues*, *Escherichia coli*, *Salmonella typhimurium* or *Shigella flexneni*.

ثم تعريضها للأوزون لأوقات صفر، 5، 10، 15، 20 أو 30 دقيقة بنفس المعدل السابق. ثم تم فورا عد سلالات البكتيريا الممرضة لمعرفة مدى بقاءها.

وقد أظهرت النتائج أن، احتواء كلا من اللبن الخام المعامل بالأوزون (لمدة أكثر من 5 دقائق) والمعامل

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