Local and Systemic Free Radicals level in Unexplained Infertile Women

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الخلاصة:

يعتبر العقم من أهم المشاكل الصحية في الدول النامية.و يمكن تحديد أسباب العقم في ٩٠% من الحالات بينما يبقى السبب في ١٠ % من الحالات غير معروف.وهذا العقم غير المفسر ربما تكمن أسبابه في الاضطرابات الفسلجية :هرمونية أو مناعية أو وراثية.

ويبدو في الوقت الحاضر إن أنواع الأوكسجين الفعالة واحدة من الأسباب الرئيسية للعقم الغير مفسر.لذلك صممت هذه الدراسة لتحديد وقياس أنواع الأوكسجين الفعالة والتي تسمى بالجذور الحرة وكذلك قياس مضادات الأكسدة في مصل الدم و الإفرازات المخاطية لعنق الرحم لمعرفة مدى علاقتها بالعقم غير المفسر.

شملت الدراسة (٦٠) امرأة مصابة بالعقم غير المفسر متوسط أعمارهن (7.195±29.866) عاما واللاتي شخصن سرريا ومختبريا. تم فحص النساء من قبل أخصائية نسائية في مستشفى بابل للولادة والأطفال وفي العيادات الخاصة في بابل و للفترة من أيلول ٢٠٠٨ ولغاية أيار ٢٠٠٩. كما تضمنت الدراسة (٣٠) عينة قياسية من النساء المنجبات متوسط أعمارهن (8.011±30.133) عاما، حيث تم دراسة نسبة ناتج الأكسدة للدهون (المالوندای الدهايد) وكذلك قياس بعض من مضادات الأكسدة (الكلوتاثايون وإنزيم الكاتاليز وفيتامين سي وفيتامين إي) في مصل الدم وفي الإفرازات المخاطية لعنق الرحم ولكلا المجموعتين.

النتائج : بينت النتائج إن نسبة المالوندای الدهايد قد ارتفعت معنويا بشكل واضح في مصل الدم و الإفرازات المخاطية لعنق الرحم(3.105 ± 1.441 و 6.729± 3.055مايكرومولاري) للنساء العقيمات عند مقارنتهم بالنساء المنجبات(١.٥٢٤ ± ٠.٥١٨ و3.201±1.028 مايكرومولاري) بالتتابع. على عكس إنزيم (الكاتاليز) حيث انخفض معنويا بشكل واضح في مصل الدم و الإفرازات المخاطية لعنق الرحم (0.303 ± 0.286 و٠.٣٣١ ±٠.٣٠٢ ك/مل) للنساء العقيمات عند مقارنتهم بالنساء المنجبات (٠.٤٧٩ ± ٠.٣٤٢ و ٠.٥٠٨ ±٠.٣٠١ ك/مل).

إما عند دراسة مضادة الأكسدة غير الإنزيمية فقد وجد انخفاض معنوي في نسبة الكلوتاثايون في مصل الدم والإفرازات المخاطية لعنق الرحم (١٧.٩٧٧±٨.٥٠٦ و ٥١.٦٠٢ ±٢٧.٣٣٥ مايكرومولاري) للنساء العقيمات عند مقارنتهم بالنساء المنجبات (٢٧.٣٨٤ ± ١٣.٥٤٧و٦٥.٣٢٧ ± ١٨.٢١٧ مايكرومولاري). كذلك نسبة فيتامين إي فقد وجد انخفاض معنوي في مصل الدم والإفرازات المخاطية لعنق الرحم (5.272± 1.228 و 4.644±1.343ملغم /لتر) للنساء العقيمات عند مقارنتهم بالنساء المنجبات (٧.٣٣٧ ±٣.٥٣٥ و٠.٧٥٤± ٧.٠٢٣ ملغم / لتر.

إما نسبة فيتامين سي فقد وجد انخفاض غير معنوي في مصل الدم و الإفرازات المخاطية لعنق الرحم (9.944±1.549و 14.233±3.458 ملغم/ لتر) للنساء العقيمات عند مقارنتهم بالنساء المنجبات (١٠.٣٨٣±٣.٦٥٥ و ١٦.٤٤٧ ± ٤.٠٤٢ ملغم /لتر).

الأستنتاج: إن أنواع الأوكسجين الفعالة واحدة من الأسباب الرئيسية للعقم وان نسبتها مرتفعة في حالات العقم الغير مفسر.

Abstract

Back ground:

Infertility is one of the most important and underappreciated reproductive health problems in developing countries.

The causes of infertility can be found in about 90% of cases, while about 10% of patients don't know why they can not conceive .The causes of this case (unexplained infertility) seems to be heterogeneous, with suggested potential causes ranging from disturbances in endocrinological, immunological , genetic and reproductive physiological factors.

One of the main causes of unexplained infertility is the reactive oxygen species (ROS). Therefore this study is aimed to estimate and calculate the ROS and to determine the relationship between free radicals, antioxidant enzymes activities and unexplained infertility. To carry out this aim, ROS has been estimated in both sera and cervical mucus secretions among healthy fertile women (control) and patients with unexplained infertility ; in form of malondialdehyde (MDA). In addition, catalase (CAT), glutathione level (GSH), vitamin C and vitamin E (as antioxidant agents) for both fertile women and infertile women studied groups have estimated and calculated.

Patients and method:

The study groups were attended to Babylon Maternity and Pediatric Hospital and privet clinics. All studied patients are suffering from primary or secondary unexplained infertility types and are diagnosed by gynecologist. The study is carried out on (30) apparently healthy fertile women as a control group, their mean age (30.133±8.011years) and (60) infertile women as patient group, their mean age (29.866 ±7.195 years).

Results : The results of the tests have shown that the malondialdehyde (MDA) were increased significantly ($p<0.01$) in serum and cervical mucus secretion(3.105± 1.441µM and 6.729±3.055)µM in patients group comparing with control group which where (1.524±0.518 µM and 3.201± 1.028µM) respectively. Catalase levels decrease significantly ($p<0.05$) in serum and cervical mucus.secretion (0.303± 0.286 k/ml and 0.331±0.302 k/ml) in patients group comparing with (0.479±0.342 k/ml and 0.508±0.301 k/ml) in control group. The glutathione levels, on the other hand, decrease significantly ($p<0.05$) in serum and cervical mucus. secretion (17.977±8.506 µM. and. 51.602± 27.335µM) in patients group comparing with (27.384 ±13.547µM and 65.327±18.217µM) in control group.Vitamin C levels decrease insignificantly ($p>0.05$) in serum and cervical mucus secretion (9.944 ± 1.549mg/l and14.233 ±3.458mg/l) in patient group comparing with (10.383±3.655mg/l and (16.447± 4.042mg/l) in control group.Vitamin E levels decrease significantly ($p<0.05$) in serum and cervical mucus secretion(5.272 ± 1.228mg/l and 4.644 ± 1.343 mg/l) in patient groups comparing with (7.337±3.535 mg/l and 7.023± 0.754 mg/l) in control groups.

Conclusion: The present study shows that the oxidative stress plays an important role in human fertility which is significantly increased in sera and cervical mucus secretion in patients with unexplained infertility.

Introduction

Reproductive failure is a significant public health concern. Infertility, carries significant personal, societal and financial consequences.One of the most important and underappreciated reproductive health problems in developing countries is the high rate of infertility and childlessness (1).

Infertility is defined as 'the inability to conceive following 12 months of unprotected sexual intercourse, before an investigation is undertaken unless medical history and physical findings dictate earlier evaluation and treatment (2). infertility is classified as primary in which no previous pregnancy has occurred and secondary in which prior pregnancy has occurred respective of its outcome(3). Causes of infertility can be found in about 90% of cases, about 10 % of patients don't know why they can not conceive this is called unexplained infertility (4).

Unexplained infertility is a diagnosis of exclusion, when the standard investigation of both the female and male partner has ruled out other infertility diagnoses (5).A couple is considered to have unexplained infertility if the woman ovulated and had a normal and hysterosalpingogram, and the man a normal semen analysis (6). Critical factors to be considered in evaluating and managing unexplained infertility are the duration of infertility and female age(4).

Because female ovary is the source of oocytes and regulating hormones, free radicals in the gynecologic environment is likely to be an important mediator of conception. Recently there is a growing evidence of possible role of highly reactive products of oxygen, termed free radicals, in infertility (7).

A free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell (1). free radicals are neutralized by an elaborate antioxidant defense system .In a healthy body, pro-oxidants and antioxidants maintain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress(8).

In this case free radical species which are unstable and highly reactive, will become stable by acquiring electrons from nucleic acids ,lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease (9). Because free radicals are unstable, and difficult to measure, traditional indices of oxidative stress include downstream markers of oxidative damage to macromolecules such as lipids, proteins and DNA. Oxidative stress is also indirectly assessed by estimating capacity for antioxidant defense in serum, or other body fluids. Such measures include assessment of enzymatic antioxidant activity and individual assessment of circulating non-enzymatic antioxidant levels (10).

Aims of the study

The aims of the present work are to explore some aspects of unexplained infertility among women. To verify this goal, the following parameters were determined:

1- The malondiadehyde (MDA) concentration in sera and cervical mucus secretions.
2- The activity of antioxidant enzyme, catalase (CAT) in sera and cervical mucus secretions.
3- The level of reduced glutathione (GSH) concentration; vitaminS C &E as non-enzymatic antioxidant in sera and cervical mucus secretions.

Patient and Methods

Patients:

Patients and control groups

Control group: This group consists of thirty apparently healthy fertile women, with a mean age (30.133±8.011years), and range from 18 to 44 years were investigated to serve as a controlling group. None of them had clinical or laboratory evidence of diseases that would affect the parameters to be measured.

Patients group: Couples resident in Babylon with unexplained infertility of more than 12 months duration were identified from the Fertility Clinic database in Babylon maternity hospital and private clinic. The patients were seen between August 2008 and May 2009.

The following criteria have been used to establish the diagnosis of unexplained infertility: mid-luteal serum progesterone concentration >20 nmol/l, bilateral tubal patency demonstrated by laparoscopy or hysterosalpingogram and normal semen parameters (4).

Sixty women had been studied, with a mean of age (29.866 ±7.195 years), and from 18 to 44 years ; 26 with primary unexplained infertility and 34 have secondary unexplained infertility. Those having male factor of infertility or female factors or any other an associated condition which could alter the level of free radicals like, hypertension, diabetes mellitus, heart disease, malignancy, and antioxidant therapy, had been excluded from the study. Each subject was involved to detailed clinical history and physical examination. The infertile group had undergone baseline investigations of infertility.

Methods:

Collection of blood and serum preparation: About 5 ml of venous blood for specific test of markers was collected by vein puncture using 5ml disposable syringes. The obtained sera was put then in another disposable tubes and labeled. The samples were transferred to the biochemical laboratory for analysis of MDA as a marker of free radicals, CAT as scavenging enzymes and vitamin C , vitamin E and GSH as non-enzymatic scavengers as well as for analysis of serum proteins level.

Collection of cervical secretion: About 0.5 ml of cervical secretion was taken by syringe from high cervix using cusco speculum, labeled and storage at – 20 C◦.For biochemical tests, the mucus must be liquefied by mucolytic agent of N-acetyl L-cycteine at concentration 0.2 mg/ml which prepared by weight 0.2 mg of N-acetyl L-cycteine and complete to one milliliter with DW (11 and 12).

Biochemical tests:

Determination of Malondialdehyde level in sera and cervical secretions

Measurement of MDA, a secondary product of lipid peroxidation, was based on the calorimetric reaction with thiobarbituric acid (TBA) to form pink color product ,which could be measured by spectrophotometer(13). :

Measurement of Catalase Activity in sera and cervical secretions

CAT was a heme enzyme that contains four ferriprotoporphyrin groups molecules and was present in peroxisomes. It catalyzes the divalent reduction of H_2O_2 (at high concentration) to water. Its activity could be induced by oxidative stresses (14**).**

Catalase activity was determined by the decrease in absorbance due to H_2O_2 conception (15).

Determination of glutathione level in sera and cervical secretions

Determination of GSH depends on the action of sulfhydryl groups(16).
Sulfhydryl group of GSH could reduce disulfide chromogen of 5,5´-Dithiobis 2.nitrobenzoic acid (DTNB) and change it to an intensely yellow compound which could measure its absorbance directly by spectrophotometer at 412 nm and it was directly proportional to the GSH concentration (17).

Determination of total vitamin C in sera and cervical secretions Numerous analytical methods were available for assessment of vitamin C nutritional status. In most methods, protein was precipitated with metaphosphoric or perchloric acid before analysis (16).
According to 2,4-dinitrophenylhydrazine (DNPH) methods, vitamin C was oxidized by Cu^{+2} to dehydroascorbic acid and diketogulonic acid (Rifal, *et al*.,1999; Boyer,2000). When treated with 2,4-DNPH, the hydrazones derivatives were produced , which, in the presence of sulfuric acid, form an orange – red complex that absorbs at 520 nm .

Determination of Vitamin E in sera and cervical secretions

Principle

After the proteins in the cervical secretion or serum are precipitated by an equal volume of absolute ethanol, the whole mixture was subjected to extraction by an equal volume of n-heptane .
The α -α dipynidyl was added to an aliquot of the upper layer to estimate the principal interfering substance, B-carotene, at 460 nm. At this time, the ferric chloride (FeCl3) reagent was added to the system to produce the colour which was measured at 510 nm(18).

Statistical Analysis

SPSS program was used in this study.All values were expressed as mean ± standard deviation (SD). Independent t-test was used to estimate differences between groups. The differences were considered significant when the probability (P) was less than 0.05($P< 0.05$)(19).

The Results:

Measurement of malondialdehyde levels:

The total MDA levels in sera and cervical secretions of infertile women with unexplained infertility shows a significant increase($p<0.001$) when compared with fertile control as shown in Table (1):

Table 1 : The total malondialdehyde levels in sera and cervical secretions in µM in infertile women with unexplained infertility and fertile Controls

Sample	Control Mean ±SD µM	Patients Mean ±SD µM	P value
Serum MDA	0.518 ± 1.524	3.105 ± 1.441	P<0.001
C.mucus MDA	3.201 ± 1.028	6.729 ± 3.055	P<0.001

Measurement of catalase enzyme activity

The catalase levels in sera and cervical secretions of infertile women with unexplained infertility shows a significant decrease ($p< 0.05$) when compared with fertile control as shown in Table (2):

Table 2: Catalase levels in sera and cervical secretions in K/ml of infertile women with unexplained infertility and Control

Sample	Control Mean ±SD **K/ml**	Patients Mean ±SD **K/ml**	P value
Serum catalase	**0.479 ± 0.342**	0.303 ± 0.286	P<0.05
C.mucus catalase	0.508 ± ٠. 301	**0.331 ± 0.302**	P<0.05

Measurement of glutathione levels

The glutathione levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease ($p< 0.001$,$P<0.05$) respectively when compared with fertile controls as shown in Table (3):

Table 3: Glutathione levels in sera and cervical secretions in µM of infertile women with unexplained infertility and fertile control

Sample	**Control Mean ±SD µM**	**Patients Mean ±SD µM**	**P value**
Serum glutathione	**27.384 ±13.547**	١٧.٩٧٧ ± ٨.٥٠٦	**P<0.05**
C.mucus glutathione	65.327± 18.217	**51.602 ± 27.335**	**P<0.001**

Measurement of vitamin C activity

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant decrease ($p>0.05$) when compared with fertile control as shown in Table (4):

Table 4: Vitamin C levels(mg/l) in sera and cervical secretions of infertile women with unexplained infertility and Control.

Sample	**Control Mean ±SD mg/l**	**Patients Mean ±SD mg/l**	**P value**
Serum Vitamin C	10.383 ± 3.655	**9.944 ± 1.549**	**P>0.05**
C.mucus Vitamin C	**16.447 ± 4.042**	14.233 ± 3.458	**P>0.05**

Measurement of vitamin E levels

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease ($p<0.05$ and $p< 0.001$) respectively when compared with fertile control as shown in Table (5):

Table 5 : Vitamin E levels in sera and cervical secretions (mg/l) of infertile women with unexplained infertility and fertile control.

Sample	**Control Mean ±SD mg/l**	**Patients Mean ±SD mg/l**	**P value**
Serum Vitamin E	**7.337 ± 3.535**	5.272 ±1 .228	p<0.05
C.mucus Vitamin E	7.023 ± 0.754	**4.644 ± 1.343**	p< 0.001

Discussion:

Malondialdehyde levels in sera and cervical secretions

The results of the present study show that the total MDA levels in sera and cervical secretions of infertile women with unexplained infertility show a significant increase ($p<0.001$) when compared with fertile controls as shown in Table (1) . The data of the present study are agreed with the results of Polak, *et al*. in 1999 (20) ; Agarwal, *et al*.in 2003 (21)and 2006 (22); as well as Savita, *et al*. in 2009 (23).

Agarwal, *et al***.** in 2005(24) suggest that women with idiopathic infertility have reduced concentrations of antioxidants in peritoneal fluid and increased ROS-induced lipid peroxidation damage resulting in infertility.

Dong, *et al*. **in** 2001(25),on the other hand, have found significantly high levels of nitric oxide in peritoneal fluid of patients with unexplained infertility.

Increased level of MDA in sera and cervical secretions in females with unexplained infertility can be attributed largely to higher rate of lipid peroxidation result in structural and functional damage to cellular membrane of both ova and transported sperms. Accumulated lipid peroxides may cause not only tissue damage but also some biological events that lead to apoptosis and accelerate termination of pregnancy (26).

Free radicals in cervical secretion and free radicals that diffuse into the fallopian tubes from peritoneal fluid may cause damage to sperm; which are known to be sensitive to OS because of their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of the scavenging enzymes (27). Therefore; ROS may affect the quality and number of spermatozoa reaching the ovum in the female reproductive tract (28).

At high concentrations, ROS may cause a series of damages to spermatozoa that include membrane lipid peroxidation, oxidation of protein, loss of motility fertilizing potential, and DNA fragmentation (29).

Therefore; spermatozoa and oocyte have built in a mechanism to prevent excessive production of ROS at the time of sperm-oocyte fusion. This may be done by the release of antioxidant enzymes (30)and if there is an abnormality in the production of antioxidant enzymes, ROS generation can continue uninterruptedly and damage both spermatozoa and oocyte.

Severe OS can lead to infertility because of the negative impact on fusion events such as acrosome reaction and sperm–oocyte fusion (28).

So the high rate of free radicals production in female with unexplained infertility may be generated from increases metabolism and depletion of protective antioxidants.

Measurement of catalase activity

Catalase level in sera and cervical secretion of infertile women with unexplained infertility show a significant decrease ($p< 0.05$) when compared with fertile controls as shown in Table (2) and this result is in agreement with the results of Pyari, *et al*. in 2006(31) who find that CAT level is significantly low in endometrium and blood of infertile women compared to those in controls. The lowest levels have been found in cases of unexplained infertility. Similarly Polak, *et al*. in 2001(20**)** find that the total antioxidant status is significantly lower in peritoneal fluid from women with unexplained infertility. In another study, wang ,*et al*. in 1997(32)find that the concentrations of antioxidants in idiopathic infertility patients significantly reduced compared to those of fertile patients .

Catalase is enzymatic antioxidant; located mainly in the tubal fluid. Neutralizes intracellular and extracellular hydrogen peroxide (1).Higher ROS levels in patients with

unexplained infertility may lead to increase ROS-scavenging process which ultimately lead to reduce levels of antioxidants such as catalase enzyme. Catalase Promotes an increase in the proportion of zygotes undergoing at least one cleavage (1). So any change in its concentration may lead to disruption of zygotes cleavage which leads to infertility.

Ali, *et al*. in 2000(33) show that the usage of catalase in media of in vitro fertilization gives better quality embryos.

Measurement of glutathione levels

Numerous laboratory studies have suggested that glutathione is a critical factor in protecting organisms against toxicity and disease. Blood and serum glutathione concentrations may serve as an indicator of GSH status, and thus diseases risk in human.

In female reproductive tract , glutathione is located mainly in the tubal fluid and oocyte/embryo. It neutralizes superoxide anion, metabolizes hydrogen peroxide and hydroxyl radical, improves the development of zygotes through the 2-cell block to the blastocyst stage, protects embryos against ROS, improves bovine embryo production, and prevents oxygen-induced embryonic malformation(1).

Studied cases of GSH levels in serum and cervical secretion of infertile women with unexplained infertility show a significant decrease ($p< 0.001, P<0.05$)respectively when compared with fertile controls. as shown in Table (3), this result agrees with the results obtained by Agarwal, *et al*.in 2008(8).

It is suggested from animal studies that the concentration of GSH in the oocyte is important to reduce the disulfide bonds during sperm nucleus decondensation and enable pronucleus(PN) formation, decapitation and pronuclear apposition (34).This is indecated by the observation that GSH antagonists disturbed the maturation of oocytes by compromising the decondensation of the sperm nucleus and thus preventing PN apposition. Furthermore, supplementation of GSH during in vitro maturation of oocytes resulted in improved male PN formation, normal fertilization and embryo development (35).

Oocytes are rich in glutathione reductase (36)**.** A glutathione deficiency can lead to instability of the mid-piece of sperm, resulting in defective motility (37). It protects plasma membrane from lipid peroxidation, scavenges superoxide and prevents O_2^- formation.

In a study consisting of infertile men , GSH led to significant improvement in the sperm quality (38).

The depletion of GSH levels in the present study, when compared with healthy control, supports the hypothesis that considers GSH a protective factor against the development of different types of diseases one of them is infertility.

Measurement of vitamin C activity

In female reproductive tract, vitamin C is located mainly in the ovary.Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant decrease ($p>0.05$) when compared with fertile controls as shown in Table (4)

Many studies indicate that the addition of ascorbic acid does prevent oocyte membrane damage and increases basement membrane turnover (39), leading to increased follicle integrity and survival. Other researchers propose that certain concentrations of alpha-tocopherol or ascorbic acid facilitate meiotic maturation of cumulus free oocytes and

can protect cumulus cell DNA damage and apoptosis (40).Supplementation inhibits follicular apoptosis and causes premature resumption of meiosis (1).

Measurement of vitamin E levels

Vitamin E is a major chain breaking antioxidant in membranes, located mainly in the ovary specially in follicular fluid.

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease ($p<0.05$;$p< 0.001$) respectively when compared with fertile controls as shown in Table (5). This result matches with the results got by Makinde and Adedeji in1994(41) and Mehendale, *et al*.in2009(42) who state that plasma vitamin E level is greater in fertile women than in infertile women.

Savita, *et al*. in 2009(23) suggest that the increased OS are associated with the decrease of antioxidants and associated with infertility.

Vitamin E directly neutralizes superoxide anion, hydrogen peroxide, and hydroxyl radical ;so increase these types of free radical may lead to depletion of vitamin E. Also vitamin E increases number of embryos developing to the expanded blastocysts and increases viability of embryos exposed to heat shock. So any change in its concentration may have a role in infertility(1).

Vitamin E may increase oocyte quality. In a human trial, infertile couples given vitamin E show a significant increase in fertility (43).

Conclusions and Recommendations

Conclusions : The present study shows that the oxidative stress plays an important role in human fertility:

- Malondialdehyde level is significantly increased in sera and cervical mucus secretion in patients with unexplained infertility.
- Catalase ,glutathione and vitamin E levels are significantly decreased in sera and cervical mucus secretion in patients with unexplained infertility.

Recommendations

- Malondialdehyde level should be carried out as primary test for assessment OS status.
- Assessment of OS as a cause of unexplained female infertility must be carried out to discriminate OS infertility from other causes of infertility.
- Studying the role of oxidants in multiple sites such as ovary, peritoneal cavity and uterus separately.
- Measurement of biomarkers of OS is subject to interlaboratory variations, and interobserver differences. A uniform method with comprehensive assessment of the OS biomarkers should be used so that the results can be compared across the studies.
- Study the role of oxidants in other types of infertility.
- Strategies to overcome OS in-vitro conditions and balancing between in vivo and in vitro environments can be utilized in assisted reproductive technique , to successfully treat infertility.

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