# Humidity and osmolality:

## Can we avoid media osmolality shifts in a dry environment?

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## Introduction

Uninterrupted culture of human embryos in dry incubators has become more common in many IVF laboratories globally, with embryos being cultured for up to 7 days without replenishment of media. Embryo culture in dry incubators is mostly carried out in microdrops of 20 – 50 µL of medium under oil to reduce evaporation; however, an oil overlay does not completely prevent evaporation, which may negatively affect embryo development and quality.[1,2] This unavoidable evaporation raises a number of questions regarding embryo culture conditions:

- What is the effect of a dry environment on the osmolality of culture medium?
- How do the different parameters of an embryo culture system affect the rate of evaporation?
- Do osmolality shifts, i.e., imbalances in the chemical composition of medium, stress the embryo, since it must undergo homeostatic regulation to adjust to its environment?
- Can this homeostatic regulation lead to metabolic adjustments that are ultimately detrimental to the growing embryo?

Published studies suggest that embryo quality, implantation rates, and ongoing pregnancy rates are improved when embryos are cultured in a humid environment. Presumably, this improvement occurs because the humid environment more closely mimics in vivo conditions.[1,3,4]

Because of these findings, Cook Medical undertook a series of experiments to better understand the effect of a dry environment under specific culture conditions. Controlling for variables, we investigated the changes in osmolality over time with different combinations of culture medium volumes and different types and volumes of oil overlay. The objective was to understand the conditions that may represent the best culture environment. **We found that in microdroplet culture systems, humidified incubators maintain the osmolality of medium over time far better than dry incubators.**

In this study, we explored the following study questions:

1. **What is the difference in medium osmolality shift between dry and humidified incubators?**
2. **Does incubating culture oil in a humidified incubator before use slow the rate of osmolality change in a dry incubator?**
3. **Are the type of oil overlay and the associated parameters of viscosity and density important in helping to slow the rise of culture medium osmolality in a dry incubator?**
4. **Does the surface-area-to-volume ratio of culture medium (SA:vol, the surface area of medium that is exposed to oil) influence the rise of medium osmolality in a dry incubator?**
5. **What is the interaction between the surface-area-to-volume ratio of culture medium and oil height relative to osmolality rise in a dry incubator?**
6. **What is the effect of the microdrop volume on osmolality rise in a dry incubator?**
7. **How does the response of microdrop culture in a humid environment compare to the experimental results in a well configuration in a dry environment?**

## Factors associated with evaporative loss

We identified a series of variables that the literature suggests are directly related to evaporative loss in culture conditions and then analyzed each one. This analysis allowed us to identify the main factors that could cause osmolality to increase in culture medium in a dry environment and to explore possible ways to diminish or eliminate these osmolality shifts that can be detrimental to optimal embryo growth.

Variables considered:

1. Incubator humidity[2,4,5]
2. Prehumidification of oil[6]
3. Oil type and properties[6,7]
4. Culture medium surface-area-to-oil interface relative to the medium volume[8]
5. Oil depth[9]

# Methods and outcomes

## 1. What is the difference in medium osmolality shift between dry and humidified incubators?

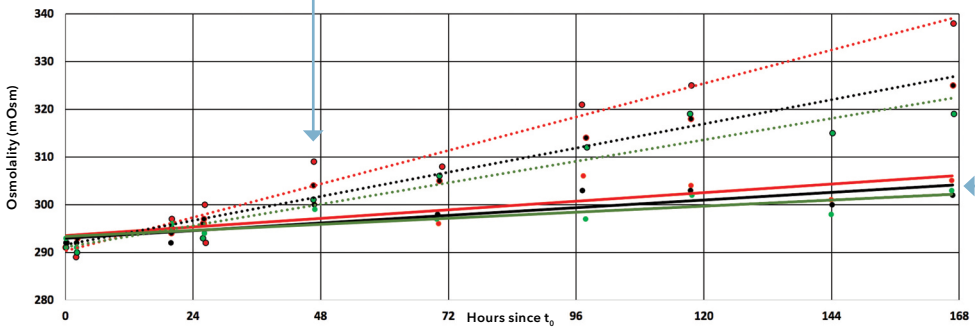
**Experiment:** Measure the rate of osmolality change in medium during continuous culture in humid vs. dry incubation conditions, using oils from three different manufacturers.

**Design:** 30 µL microdrops of Cook Sydney IVF Cleavage Medium were placed in 35 mm dishes, overlaid with 3 mL of oil, and cultured at 37 °C in dry or humid incubator conditions.

**Main points of consideration:**

- Dry incubator ≈ 10% relative humidity
- Humidified incubator ≈ 80% relative humidity
- Increases in osmolality can negatively influence embryo development[2]

Even with the "best" oil, osmolality reached 300 mOsm at day 2 in dry conditions vs. day 5 in humid conditions




No statistical difference between the slopes of the 3 different oils under humid conditions

| Oil code | Humidity condition | Density at 37 °C (g/mL) | Kinematic viscosity 37 °C ($mm^2$/sec) | Slope parameter (mOsm/hr) | Osmolality change (mOsm/24 hr) |
|---|---|---|---|---|---|
| 1 | Dry | 0.8252 | 10.57 | 0.292 | 7.0 |
| 2 | Dry | 0.838 | 14.86 | 0.211 | 5.1 |
| 3 | Dry | 0.8464 | 60.3 | 0.187 | 4.5 |
| 1 | Humid | 0.8252 | 10.57 | 0.075 | 1.8 |
| 2 | Humid | 0.838 | 14.86 | 0.067 | 1.6 |
| 3 | Humid | 0.8464 | 60.3 | 0.053 | 1.3 |

Daily rate of osmolality change is much greater under dry than humid conditions

**Results**

**Dry conditions (dotted lines)**

- A statistically significant rate of increase in osmolality occurs over time (4.5 to 7.0 mOsm per day).
- The oil type affects the rate of change of medium osmolality.

**Humid conditions (solid lines)**

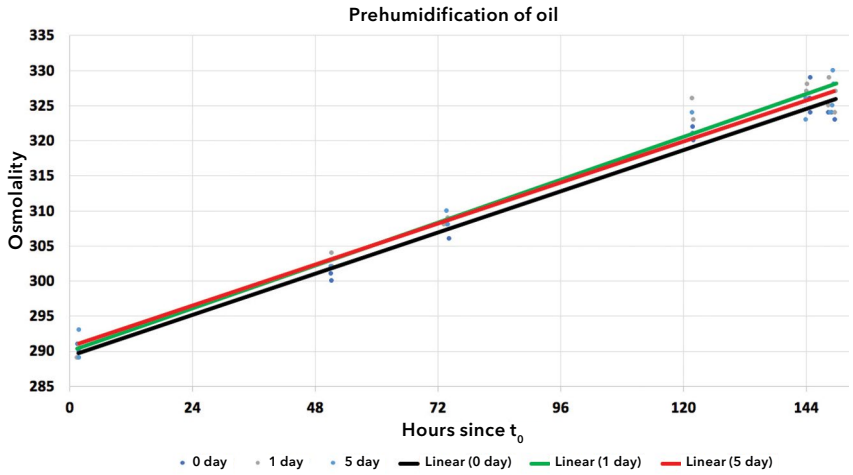
- The three oil brands had similar rates of osmolality increase: just under 2 mOsm per day.
- The type of oil doesn't have a strong effect on the rise of osmolality in the medium.
- The expected osmolality value at any time = Starting osmolality + 0.065 * Hours since $t_0$.

**Conclusion of experiment 1: Humid incubation is superior to dry incubation in moderating osmolality rise, regardless of oil type.**

## 2. Does incubating culture oil in a humidified incubator before use slow the rate of osmolality change in a dry incubator?

**Experiment:** Measure the rate of osmolality change during continuous culture in dry conditions after prehumidification of oil (0, 1, or 5 days).

**Design:** 30 µL microdrops of Cook Sydney IVF Cleavage Medium were placed in 35 mm dishes, overlaid with 3 mL of oil (either prehumidified oil or, as a control, oil without prehumidification), and cultured at 37 °C in dry incubator conditions.




**Results**

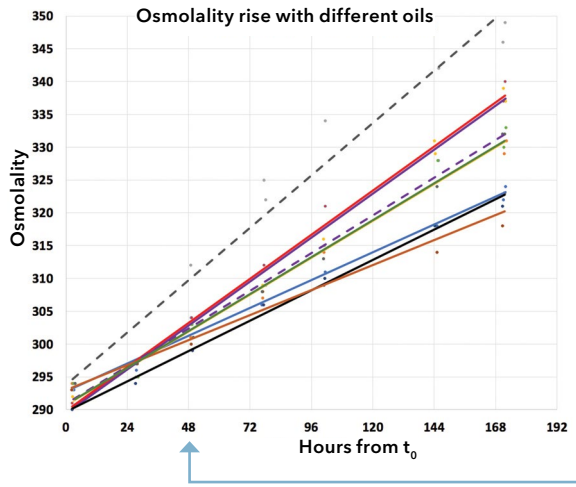
- Prehumidification of oil did not show an improvement in mitigating osmolality changes in a dry environment.
- The duration of prehumidification did not influence the results.

**Conclusion of experiment 2: Oil humidification shows no benefit on rate of osmolality change.**

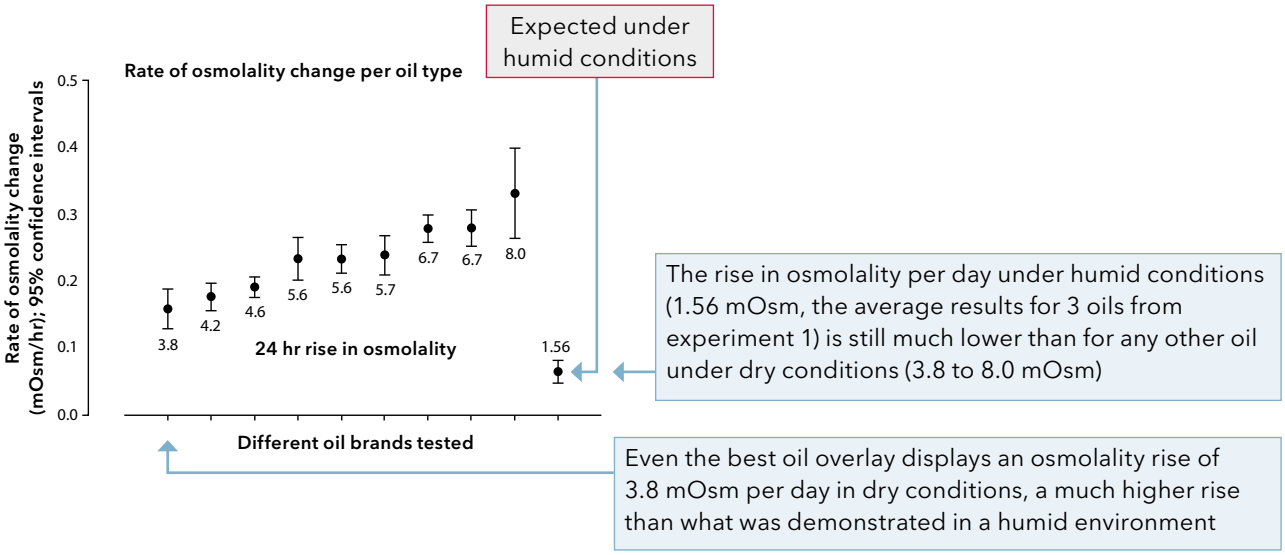
## 3. Are the type of oil overlay and the associated parameters of viscosity and density important in helping to slow the rise of culture medium osmolality in a dry incubator?

**Experiment:** Measure the effect of 9 oils from different manufacturers (7 brands and 9 oil types with various viscosities and densities) on the rate of osmolality change in medium under dry conditions during continuous culture.

**Design:** 30 µL microdrops of Cook Sydney IVF Cleavage Medium were placed in 35 mm dishes, overlaid with 3 mL of oil, and cultured at 37 °C in dry incubator conditions.




300 mOsm was reached at day 2, regardless of the type of oil used




**Results**

- Oil density ranged from 0.824 to 0.854 g/mL, and kinematic viscosity ranged from 8.84 to 79.50 $mm^2$/sec. Both oil density and viscosity are predictors of osmolality change: As density and/or viscosity increase, the rise in medium osmolality per hour under dry conditions slows.
- Oil density has a tightly correlated linear relationship to medium osmolality shifts ($R^2 = 0.91$).
- Regardless of the type or brand of oil used, a humid environment is still more stable.

**Conclusion of experiment 3: While the type of oil used does influence the rate of osmolality drift, no oil that was tested could fully compensate for the dry incubator conditions.**

## 4. Does the surface-area-to-volume ratio of culture medium (SA:vol, the surface area of medium that is exposed to oil) influence the rise of medium osmolality in a dry incubator?

**Design:** 96-well plates (Corning) and 5-well plates (Minitube) were used. In order to allow for fixed geometry and easier experimental control, the culture medium was not in droplet form. A 96-well plate was chosen because the surface area of medium in a 96-well plate is most similar to that of 20 to 50 µL microdrops under oil.

**Main points of consideration:**

- When microdrops are being prepared for embryo culture with an overlay of oil, the surface area of culture medium that is exposed to the oil overlay is directly related to the drop volume.
- Microdrops of medium are not identical, because of variations in embryologists' technique and other factors.
- The two previous points make it difficult to systematically vary the surface-area-to-volume ratio. If the geometry of the surface area is fixed (e.g., circular geometry in a well), then the volume of medium can be altered while the amount of surface area is fixed, allowing for experimental manipulation of the SA:vol.

**Results**

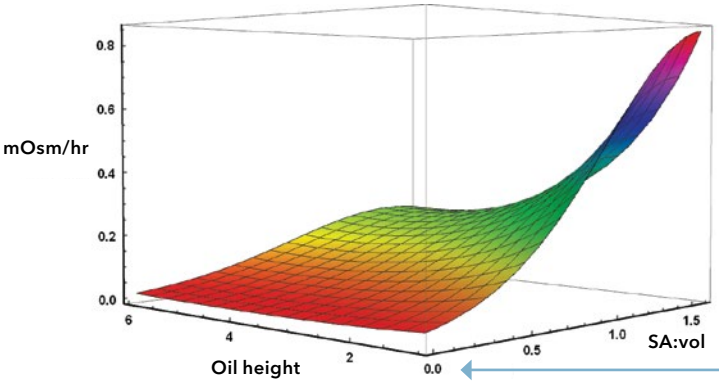
- The biggest effect was observed when the medium volume was lowest and the SA:vol was the highest. The smaller volume of culture medium caused a faster rate of evaporation and osmolality rise.
- For identical volumes of medium, the evaporation rate is higher if the surface area is more spread out (e.g., in a 5-well dish instead of a 96-well dish).
- The higher the surface-area-to-volume ratio, the faster the osmolality rise (mOsm/hour).

**Conclusion of experiment 4: The higher the surface-area-to-volume-ratio of culture medium, the faster the osmolality rises under dry incubator conditions.**

## 5. What is the interaction between the surface-area-to-volume ratio of culture medium and the oil height relative to osmolality change in a dry incubator?

**Design:** Analysis of surface plots with combined data for SA:vol and oil height.

- The surface plot represents the osmolality rise of culture medium in a dry incubator
- Oil height is defined as the thickness of the oil above the medium's surface




As medium SA:vol goes up and oil height goes down, the osmolality rise is much faster

Where medium SA:vol is low, the effect of oil height is minimal

**Results**

- Osmolality shifts are dramatically different between culture media with 1 – 6 mm oil overlays and surface-area-to-volume ratios of 0.5 – 1.5.
- A thinner layer of oil is much less effective at reducing osmolality rise at increased surface-area-to-volume ratios.

**Conclusion of experiment 5: Culture medium surface-area-to-volume ratio and oil height combine to affect culture medium osmolality change.**

## 6. What is the effect of the microdrop volume on osmolality rise in a dry incubator?

**Experiment:** Measure the effect of culture medium volume on osmolality rise (mOsm/hour) in a dry environment.

**Design:** 25 µL microdrops of Cook Sydney IVF Cleavage Medium were used for humidified conditions and 25 µL, 30 µL, 40 µL, and 50 µL microdrops for dry conditions. All media were cultured in 35 mm dishes, overlaid with 3 mL of oil, and cultured at 37 °C. Microdrop surface area measurements were performed microscopically with quantitative image analysis, and SA:vol values were calculated. The surface area values of the 25 µL microdrops were estimated by use of interpolation. (20 µL values were measured but not shown.)

| Microdrop size | SA:vol | mOsm/hr | mOsm/day |
|---|---|---|---|
| 25 µL, dry | ≈ 1.12:1 | 0.29 | 6.96 |
| 25 µL, humid | ≈ 1.12:1 | 0.07 | 1.68 |
| 30 µL, dry | 1.07:1 | 0.27 | 6.57 |
| 40 µL, dry | 1.03:1 | 0.22 | 5.42 |
| 50 µL, dry | 1.03:1 | 0.22 | 5.49 |

Increasing microdrop volume is beneficial in a dry environment but is still not good enough to obtain the same effect as culturing in a humid environment. (Osmolality change occurs about 4 times faster.)

**Results**

- The change in osmolality is fastest with a small drop volume.
- Under microdrop conditions, the change in osmolality is much faster in a dry environment.

**Conclusion of experiment 6: Microdrop culture in dry conditions results in rapid changes in osmolality over time. Culturing microdrops in a dry condition does not limit osmolality shift as well as culturing in humid conditions.**

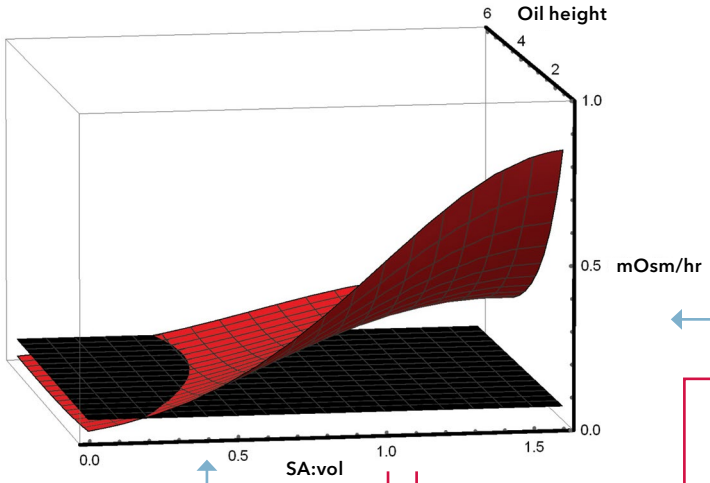
## 7. How does the response of microdrop culture in a humid environment compare to the experimental results in a well configuration in a dry environment?

**Design:** Surface plots and combined data from the previous experiments with microdrops were analyzed further.

- The red surface plot represents the rise of culture medium osmolality in a dry incubator.
- The black surface represents the expected rise of culture medium osmolality in humid conditions with microdrop culture.
- Oil height for microdrops is defined as the thickness from the bottom of the dish in mm.

**Main points of consideration:**

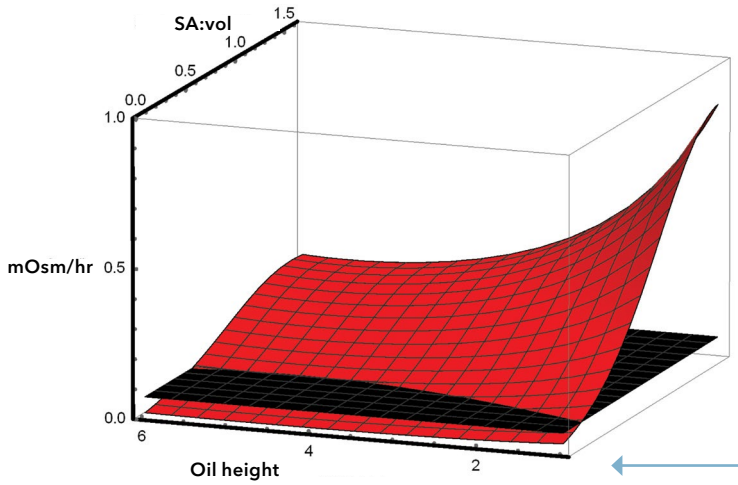
- 1 mL of oil in a 35 mm dish is approximately 1.1 mm thick.
- The SA:vol parameter can be used to compare microdrop culture and 96-well culture.
- The experiment with microdrops used 3 mm of oil, the average value used in the 96-well study.
- Regardless of whether the medium is in a microdrop or well configuration, the osmolality responses are nearly identical for the same conditions of SA:vol and oil thickness.




Culturing in a dry environment does not reduce the osmolality shift over time as much as culturing in a humid environment

SA:vol region associated with microdrop culture

The intersection of the red plot and black plot represents the region at which similar rates of osmolality rise in a dry vs. humid environment are expected to be seen, which is quite low at a SA:vol ≈ 0.4:1




A fairly high level of oil is required to retard the rate of osmolality change in a dry environment in comparison to a humid environment

**Results**

- When the SA:vol is greater than 0.4:1, the rate of osmolality change is greater in a dry environment than a humidified environment.
- A high level of oil (≥ 4 mm) is required in order to limit the osmolality change at a high SA:vol.
- At surface-area-to-volume ratios associated with microdrops, dry culture conditions result in a much higher rate of osmolality change than humid conditions.

**Conclusion of experiment 7: Under microdrop conditions, oil height (up to 6 mm) can never compensate for low humidity. Surface-area-to-volume ratios associated with dry microdrop culture result in a dramatic increase in osmolality over time.**

## Discussion

Over a number of experiments and under a large number of conditions, we have shown that incubation of culture medium in a dry incubator results in a surprising increase in medium osmolality over time, which can be detrimental to cell viability. However, osmolality, per se, is not the only thing to worry about. In the development of any particular culture medium, each component is set at an optimal concentration for that medium, and the various components and associated concentrations have an interaction effect. If osmolality increases, the concentration of each constituent increases in proportion to the starting concentration. Because of a chemical effect, this increase in the concentration of each constituent may have a larger effect on cell viability than the associated increase in osmolality. As a result, changes in constituent concentrations, even within what is considered to be a "normal" osmolality range, may cause the culture medium to perform worse. This chemical effect is likely the explanation for why different culture systems with different initial osmolality values have shown reduced performance in a dry environment.[1,3,4]

We encourage continual validation of the culture system as part of the laboratory quality management system. Possible culture medium evaporation and the associated osmolality increase should be looked at more closely to avoid unwanted negative outcomes, especially for uninterrupted culture systems in dry incubators.

## Conclusion

This set of experiments enabled Cook Medical to develop a mathematical model that uses the variables we identified, i.e., culture medium surface-area-to-volume ratio and oil thickness, to quantify medium osmolality rise in an incubator over time. This predictive model allowed us to modify any individual factor and assess the influence on the system's osmolality change per hour.

Exploring the various factors that can influence the osmolality of the medium in a culture system and thus influence the development of the embryos in culture has provided insight into how to best manage this potential risk. In a microdrop culture system, the risk is best managed through the use of humidified incubators, which maintain the osmolality of medium over time better than dry incubators.

## Summary of results

| Study question | Summary conclusion |
|---|---|
| What is the difference in medium osmolality shift between dry and humidified incubators? | Humid incubation results in a shift in osmolality of about 10 mOsm over 7 days. This shift is as high as 56 mOsm over the same period in dry conditions. |
| Does incubating culture oil in a humidified incubator before use slow the rate of osmolality change in a dry incubator? | Oil preincubation in a humid environment shows no benefit on the rate of osmolality change when the oil is then used in a nonhumidified culture environment. |
| Is the type of oil overlay important in helping lower culture-medium osmolality rise in a dry incubator? | While the type of oil used does influence the rate of osmolality rise, no oil that was tested could fully compensate for the dry incubator conditions. |
| Does the culture medium's surface-area-to-volume ratio influence medium osmolality rise in a dry incubator? | A higher culture medium surface-area-to-volume ratio causes a faster rise in medium osmolality. |
| What is the interaction between the surface-area-to-volume ratio of culture medium and oil height relative to osmolality rise in a dry incubator? | Culture medium surface-area-to-volume ratio and oil height both affect culture medium osmolality change. Thinner oil overlays and higher surface-area-to-volume ratios accelerate osmolality rise. |
| What is the effect of microdrop volume on osmolality rise in a dry incubator? | Smaller microdrops have a larger surface-area-to-volume ratio, which results in a higher rate of osmolality change in nonhumidified culture conditions. |
| How does the response of microdrop culture in a humid environment compare to the experimental results in a well configuration in a dry environment? | Under microdrop conditions, oil height (up to 6 mm) can never compensate for low humidity. Surface-area-to-volume ratios associated with dry microdrop culture result in a dramatic increase in osmolality over time. |

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