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ORIGINAL ARTICLE ***Infertility***

Treatment with Ca^{2+} ionophore improves embryo development and outcome in cases with previous developmental problems: a prospective multicenter study

T. Ebner[1,2,*], P. Oppelt[1,2], M. Wöber[3], P. Staples[4], R.B. Mayer[1,2], U. Sonnleitner[5], S. Bulfon-Vogl[6], I. Gruber[7], A.E. Haid[1,2], and O. Shebl[1,2]

[1]Department of Gynecological Endocrinology and Kinderwunsch Zentrum, Landes-Frauen-und Kinderklinik, Krankenhausstr. 26-30, A-4020 Linz, Austria [2]Faculty of Medicine, Johannes Kepler University, 4020 Linz, Austria [3]Kinderwunschzentrum Goldenes Kreuz, 1090 Vienna, Austria [4]Medical University, 8010 Graz, Austria [5]Babywunsch-Klinik Dr. Zajc, 5020 Salzburg, Austria [6]Sterignost, Institut für Kinderwunschbehandlung, 9020 Klagenfurt, Austria [7]Kinderwunschambulanz im Landesklinikum St Pölten, 3100 St Pölten, Austria

*Correspondence address. E-mail: thomas.ebner@gespag.at

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STUDY QUESTION: Does calcium ionophore treatment (A23187, calcimycin) improve embryo development and outcome in patients with a history of developmental problems/arrest?

SUMMARY ANSWER: Application of A23187 leads to increased rates of cleavage to 2-cell stage, blastocyst formation and clinical pregnancy/live birth.

WHAT IS KNOWN ALREADY: Studies on lower animals indicate that changes in intracellular free calcium trigger and regulate the events of cell division. In humans, calcium fluctuations were detected with a peak shortly before cell division. Interestingly, these calcium oscillations disappeared in arrested embryos. Mitotic division blocked with a Ca^{2+} chelator could be restored by means of ionophores in an animal model.

STUDY DESIGN, SIZE, DURATION: This prospective, multicenter (five Austrian centers), uncontrolled intervention study (duration 1 year) includes 57 patients who provided informed consent.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Inclusion criteria were complete embryo developmental arrest in a previous cycle (no transfer), complete developmental delay (no morula/blastocyst on Day 5), or reduced blastocyst formation on Day 5 ($\leq$15%). Severe male factor patients and patients with $<$30% fertilization rate after ICSI were excluded because these would be routine indications for ionophore usage. The total of the 57 immediately preceding cycles in the same patients constituted the control cycles/control group. In the treatment cycles, all metaphase II-oocytes were exposed to a commercially available ready-to-use ionophore for 15 min immediately after ICSI. After a three-step washing procedure, *in vitro* culture was performed as in the control cycles, up to blastocyst stage when achievable.

MAIN RESULTS AND THE ROLE OF CHANCE: Fertilization rate did not differ (75.4 versus 73.2%); however, further cleavage to 2-cell stage was significantly higher ($P < 0.001$) in the ionophore group (98.5%) when compared with the control cycles (91.9%). In addition, significantly more ($P < 0.05$) blastocysts formed on Day 5 in the study compared with the control group (47.6 versus 5.5%, respectively) and this was associated with a significant increase ($P < 0.01$) in the rates of implantation (44.4 versus 12.5%), clinical pregnancy (45.1 versus 12.8%) and live birth (45.1 versus 12.8%). All babies born at the time of writing (22/28) were healthy.

LIMITATIONS, REASONS FOR CAUTION: The frequency of patients showing embryo developmental problems was expected to be low; therefore, a multicenter approach was chosen in order to increase sample size. In one-third of the cycles, the clinician or patient requested a change of stimulation protocol; however, this did not influence the developmental rate of embryos.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first evidence that developmental incompetence of embryos is an additional indication for ionophore treatment. The present approach is exclusively for overcoming cleavage arrest.

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Key words: blastocyst formation / calcium ionophore / cleavage to 2-cell stage / developmental arrest / developmental delay

Introduction

In vivo, a sperm-borne phospholipase C ζ (PLCζ) has been identified as the physiological agent of oocyte activation (Saunders *et al.*, 2002). Normally, this factor enters the ooplasm and cleaves membrane-bound phosphatidylinositol biphosphate (PIP_2) yielding diacylglycerol (initiates zona reaction) and inositol triphosphate (IP_3). IP_3 subsequently binds to its receptors located at the endoplasmic reticulum which causes calcium release from this internal store (Berridge, 2009). The resulting Ca^{2+} flux presents in an oscillatory mode. Any deficiency in these crucial biochemical substances (e.g. PLCζ, PIP_2, IP_3) will automatically cause a reduction in intracellular calcium, in particular the absence of Ca^{2+} oscillations. This obvious drawback can be compensated by artificially increasing calcium in the oocyte and, thus, inducing oocyte activation.

In the case of oocyte- and/or sperm-related fertilization problems after ICSI, methods such as electrical oocyte activation (Yanagida *et al.*, 1999; Mansour *et al.*, 2009; Baltacı *et al.*, 2010) or modified ICSI techniques (Tesarik *et al.*, 2002; Ebner *et al.*, 2004) have successfully been applied to rescue oocyte activation. This artificial process is achieved using a variety of chemical agents, with calcium ionophores, such as ionomycin or calcimycin (A23187), leading the way (Heindryckx *et al.*, 2008; Vanden Meerschaut *et al.*, 2012).

In order to fulfill the wide range of functions, Ca^{2+} levels need to be both flexible and precisely regulated, and this high versatility is achieved by various changes in signal timing and amplitude. Since overexposure to Ca^{2+} may lead to cell death, cells rather use low-amplitude Ca^{2+} signals or provide the trigger as brief Ca^{2+} transients. If the information has to be passed on over longer time periods, as is the case during oocyte activation, repetitive Ca^{2+} signals in the form of oscillations are used which will cease after pronuclear formation (for review, see Berridge *et al.*, 1998).

In vivo and *in vitro*, there are no further changes to Ca^{2+} until the zygote prepares for the first cell division, when a spontaneous Ca^{2+} signal arises driving cleavage to 2-cell stage. There is evidence that—apart from this main Ca^{2+} trigger—oscillating levels of proteins are involved in mitosis (Swanson *et al.*, 1997). For subsequent cleavages, a close correlation between cell division and Ca^{2+} availability has also been reported (Berridge, 1995).

Alterations in calcium signaling (Vanden Meerschaut *et al.*, 2013) may be the underlying reasons for defects in cell growth and cleavage (Berridge *et al.*, 1998). In humans, Sousa *et al.* (1996) have shown that sinusoidal Ca^{2+} fluctuations observed shortly before every mitotic division disappeared progressively in arrested human embryos. This observation is in line with findings of Wong *et al.* (2005) who could artificially stop cleavage in *Drosophila melanogaster* by using Ca^{2+} chelators. Application of an ionophore (ionomycin and calcimycin), however, did overcome negative effects that are known to be caused by Ca^{2+}-deficiency, e.g. cleavage furrow regression (Wong *et al.*, 2005).

These data led us to consider a treatment with A23187 (calcimycin) in patients suffering from developmental problems of their embryos *in vitro* in a previous ICSI cycle.

Materials and Methods

After receipt of ethical approval (D-17-13; Ethics Committee Upper Austria), five Austrian centers prospectively recruited patients who had either

(i) complete developmental arrest (no transfer),
(ii) complete developmental delay (no morula/blastocyst on Day 5), or
(iii) significantly reduced blastocyst formation (≤15%) in a previous cycle.

A total of 57 patients [mean (± SD) age: 32.7 ± 4.5 years, median: 32 years, range: 24–41 years] could be enrolled in the prospective analysis. Informed consent was obtained from all patients. The vast majority of patients suffered from tubal (28%) or male factor infertility (47%). The remaining patients had either endometriosis (15%) or polycystic ovary syndrome (10%). With respect to the three inclusion cohorts, it turned out that patients were equally distributed ($n = 18$, 19 and 20 for Groups A, B and C, respectively). In order to ensure a prospective approach, all cycles were reported to the principal investigator (T.E.) on the day of oocyte collection. Each patient was only included once.

Severe male factor patients and patients with <30% fertilization rate after ICSI were excluded because these are routine indications for ionophore usage (Ebner *et al.*, 2012, 2014; Montag *et al.*, 2012). The immediately preceding conventional ICSI cycles (all within a 6-month period of the study cycle) served as the control group, although 23 patients had more than one previous cycle showing developmental incompetence.

In the treatment cycles under study, all metaphase II (MII) oocytes were exposed to a commercially available ready-to-use ionophore (GM508 Cult-Active; Gynemed, Lensahn, Germany) for 15 min immediately after ICSI. After a three-step washing procedure, *in vitro* culture was performed as in the control cycles, when possible up to blastocyst stage. It should be emphasized that according to our ethics committee vote it was for the clinician or the patient to choose the preferred type of stimulation protocol. This led to a change in stimulation protocol of the study cycle between agonist and antagonist protocol (and vice versa) in 18 cases (31.6%).

In the present study, all female partners were either stimulated according to a long protocol including down-regulation with the GnRH-agonist Triptorelin (Ferring, Vienna, Austria) or buserelin (Sanofi-Aventis, Frankfurt, Germany), or according to an antagonist protocol using Cetrorelix (Merck Serono, Vienna, Austria) or Ganirelix (MSD). Gonadotrophins used in both regimens were of recombinant (Puregon, MSD) and/or urinary origin (Menopur, Ferring, Vienna, Austria). Oocyte collection was scheduled 36 h after ovulation induction with 10 000 IU β-hCG (Ovitrelle, Merck Serono).

All interventions, such as sperm collection and preparation, oocyte retrieval, ICSI, embryo culture and transfer, were performed according to the standard protocol of the individual centers; however, all centers worked with sequential media and group culture using smaller volumes (30–50 μl).

Quality assessment of oocytes, zygotes and embryos was performed according to previously published guidelines (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Particular attention was given to cleavage stage embryos, thus reflecting mitotic activity. According to the above-mentioned guidelines, embryo quality was checked for stage appropriateness (expected stage of development

at a given time of observation) and quality (good, fair and poor quality based on fragmentation, multinucleation, and cell size). Days 2, 3 and 4 embryo assessment was performed at 43–45, 67–69 and 91–93 h post-injection, respectively.

In addition, survival rate was calculated as the percentage of embryos that still showed mitotic activity at the scheduled day of embryo transfer (e.g. that had at least one mitotic division between the day before transfer and transfer day). With respect to this, it should be clarified that two patients in the control group (Indication A) had one embryo each which was not transferred, although mitosis was still ongoing. This was the joint decision of clinicians, embryologists and patients due to the fact that both embryos showed temporary arrest (24 h) followed by one subsequent cleavage. These embryos were referred to 'Indication A' since (i) the majority of the sibling embryos already had stopped development, and (ii) neither of these two embryos showed further cleavage the day after canceled embryo transfer.

In principle, a transfer at blastocyst stage (Day 5) was striven for; however, every fifth patient ($n = 12$) had the transfer at cleavage stage (Day 3 or 4). The mean ($\pm$SD) number of embryos/blastocysts transferred was 1.4 ($\pm$0.5). Four patients had all their blastocysts vitrified due to ovarian hyperstimulation syndrome.

Statistics

To assess differences between the study and control group, we performed a repeated measures multivariate analysis of variance. As within-subject factors, ionophore treatment (measures: fertilization rate, survival rate, cleavage rate) and interaction factors were considered. The corresponding between-subject factors were stimulation regime [long versus antagonist protocol), Indication (A–C) and interaction factors]. In the case of significance, we additionally performed an univariate analysis and *post hoc* test of between-subject effects. The factor 'blastulation rate' was excluded from the repeated-measures multivariate analysis as the number of subjects in the historic control group was too low (*cave*: reduced blastulation was an inclusion criterion). However, we performed a repeated-measures analysis of variance (within-subject factor and between-subject factors as above). Since the number of subjects in this study was limited, we performed *post hoc* power and effect size analyses and present the results for significant differences. Changes in dichotomous variables (e.g. transfer cancellation) due to the treatment were assessed by the McNemar tests.

We defined the significance level as $P < 0.05$. Multi- and univariate analysis as well as McNemar tests were performed using SPSS22, and power analysis was performed using G*Power (Faul *et al.*, 2009).

Results

Historical control cycles resulted in 594 cumulus–oocyte complexes (COC) and 489 mature MII gametes. The associated ICSI led to 358 fertilizations (73.2%). In 57 study cycles, a total of 604 COCs were collected. These contained 512 MII gametes; of which, 386 (75.4%) could be fertilized.

However, the next day less oocytes cleaved to the 2-cell stage in the control group (91.9%) when compared with the ionophore-treated group (98.5%). This was especially true for Groups B and C as indicated in Table I ($P = 0.054$). This observed developmental deficiency was found throughout preimplantation development since a significantly lower percentage of embryos showed mitotic activity on the day of embryo transfer (=survival rate) in the control cycles. Table II indicates that at the cleavage stage, mitotic rate but not embryo morphology (except Day 4) was significantly better in ionophore cycles when compared with control ones.

The effect of ionophore treatment measured by blastulation rate revealed a significant difference [$P = 0.039$, effect size = 1.08 (large), power = 1 (perfect)], indicating an improvement of blastulation rate by the treatment. However, these results are preliminary indications as the number of subjects in the control group is low.

In terms of outcome, it was evident that significantly fewer cycles were canceled in the ionophore group as highlighted in Table III. Not only a significant shift ($P < 0.001$) from the cleavage stage to blastocyst transfers was observed but also significantly increased rates of implantation ($P < 0.001$), clinical pregnancy ($P < 0.01$) and live birth ($P < 0.01$) in the study cohort (Table II). Significantly, more embryos were vitrified in the ionophore group when compared with the historical control cycles (74 versus 6) which led to a significant improvement in embryo utilization rate ($P < 0.001$). All children born so far in the study and control group are healthy.

Table I Comparison of preimplantation development between the study (calcium ionophore treatment) and control group (conventional ICSI), subdivided by indication.

	MII	2PN	Cleavage	Survival[a]	Blastulation[b]
Ionophore cycle					
Group A	138	105 (76.1)	103 (98.1)	49 (47.6)	35/73 (48.0)
Group B	187	137 (73.3)	135 (98.5)	83 (61.5)	62/123 (50.4)
Group C	187	144 (77.0)	142 (98.6)	81 (57.0)	62/138 (44.9)
Control cycle					
Group A	122	85 (69.7)	83 (97.7)	2 (2.4)	0/55 (0)
Group B	156	109 (69.9)	97 (89.0)	31 (32.0)	0/102 (0)
Group C	211	164 (77.7)	149 (90.9)	30 (20.1)	18/153 (11.8)

MII, metaphase II; PN, pronucleii.
McNemar test.
Values in parentheses are percentages. Blastulation is calculated as blastocyst per 2Pn cultured on Day 5. Cleavage is assessed as cleavage to 2-cell stage. Survival refers to residual mitotic activity on day of transfer.
Group A, developmental arrest; Group B, developmental delay; Group C, reduced blastulation.
[a,b]$P < 0.01$ (study group compared with the corresponding control group).

Table II Cleavage stage morphology (Days 2–4) of study and control cycles based on cell number and morphology, according to the Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology (2011).

	Ionophore cycle			Control cycle		
	A	B	C	A	B	C
D2 cleavages (*n*)	103	135	142	83	97	149
D2 cell number[a]	70 (68.0)	111 (82.2)	109 (76.7)	38 (45.8)	52 (53.6)	80 (53.7)
D2 good quality	44 (42.7)	79 (58.5)	73 (51.4)	32 (38.6)	58 (59.8)	68 (45.6)
D3 cell number[a]	60 (58.3)	86 (63.7)	84 (59.2)	14 (16.9)	36 (37.1)	58 (38.9)
D3 good quality	45 (43.7)	84 (62.2)	68 (47.9)	21 (25.3)	50 (51.6)	65 (43.6)
D4 cell number[a]	46 (44.7)	71 (52.6)	71 (50.0)	2 (2.4)	16 (16.5)	33 (22.2)
D4 good quality[a]	29 (28.2)	70 (51.9)	69 (48.6)	1 (1.2)	4 (4.1)	54 (36.2)

Values in parentheses are percentages.
Cell number refers to number of ≥4-cell (Day 2), ≥8-cell (Day 3) and compacting embryos (Day 4), respectively. Good quality embryos at any stage are characterized by <10% fragmentation, absence of multinucleation and appropriate cell size.
McNemar test.
[a]$P < 0.001$ (ionophore cycle versus control cycle).

Table III Comparison of treatment outcome between study (calcium ionophore treatment) and control group (conventional ICSI).

	Ionophore cycle	Control cycle	*P*-value
n cycle	57	57	
n canceled transfers	2 (3.5)	18 (31.6)	0.02
n OHSS	4 (7.0)	1 (1.8)	0.375
n blastocyst transfer (Day 5)	38	11	<0.001
Mean *n* of embryos transferred	1.3 ± 0.6	1.2 ± 0.4	
Positive β-hCG	28/51 (54.9)	6/39 (15.4)	<0.001
Miscarriage (negative HA)	2 (7.1)	0	0.50
Miscarriage (positive HA)	3 (10.7)	1 (16.7)	0.25
Clinical pregnancy	23 (45.1)	5 (12.8)	0.01
Implantation rate	32/72 (44.4)	6/48 (12.5)	<0.001
Multiple pregnancy rate	7/28 (25.0)	0	0.063
Live birth	23 (45.1)	5 (12.8)	0.01
Children born	18/28	5	
Malformation	0	0	

Values in parentheses are percentages. OHSS (ovarian hyperstimulation syndrome): no fresh transfer. Multiple pregnancies include two monozygotic twins and one miscarriage of twins. HA, heart activity.
McNemar test.

The multivariate analysis showed a significant multivariate effect for the combined dependent variables fertilization rate, survival rate and cleavage rate with respect to the type of treatment ($P < 0.001$) [effect size = 1.4 (large), power = 1 (perfect)]. No significant interaction effect was observed, indicating that neither Indication (A–C) nor stimulation regimen (long versus antagonist protocol) interfere with the results.

The subsequent univariate analysis revealed that fertilization rate did not differ significantly with respect to treatment ($P = 0.751$), whereas survival rate as well as cleavage to 2-cell stage improved significantly ($P < 0.001$; effect size = 1.3, power = 0.9999 and $P = 0.003$; effect size = 0.43, power = 0.408, respectively).

Discussion

One of the most universal signaling agents in human cells is the calcium ion. At the cellular level, Ca^{2+} originates from two sources—one external and one internal. Either it is released from internal stores through channels in the endoplasmic reticulum or it enters the cell via sensitive channels that bridge the oolemma. Ca^{2+} is only available to a limited extent and once internal stores are deficient or emptied the oocyte/embryo will be reliant on influx of extracellular Ca^{2+} (Arnoult *et al.*, 1996; Berridge *et al.*, 1998). Ca^{2+} ionophores facilitate such mechanisms.

When it comes to keeping intracellular calcium level sufficiently high, most embryologists rely on the use of Ca^{2+} ionophores, such as ionomycin or calcimycin (A23187). Except for some authors having applied these agents for activating oocytes parthenogenetically (Liu *et al.*, 2014; Parmegiani *et al.*, 2014) or for correcting the pronuclear position within the ooplasm (Isachenko *et al.*, 2010), a limited number of indications that would require ionophore treatment have emerged.

Over the past 20 years, Ca^{2+}-ionophores have been successfully used in cases of complete globozoospermia (Rybouchkin *et al.*, 1997; Tejera *et al.*, 2008; Kyono *et al.*, 2009; Taylor *et al.*, 2010) or other severe forms of isolated teratozoospermia (Moaz *et al.*, 2006; Nasr-Esfahani *et al.*, 2008). Further evidence that male factor infertility is the main indication for Ca^{2+}-ionophore treatment comes from studies dealing with crypto- (Ebner *et al.*, 2012) or azoospermia (Borges *et al.*, 2008, 2009; Stecher *et al.*, 2011; Ebner *et al.*, 2012).

However, even in the presence of a normally shaped spermatozoon, ICSI does not always guarantee successful fertilization, making failed or impaired fertilization after ICSI, the second indication for routine ionophore application. Pertaining case reports (Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004; Murase *et al.*, 2004) were followed by larger retro- (Montag *et al.*, 2012) and prospective studies (Ebner *et al.*, 2014), suggesting the presence of a 50% previous ICSI fertilization rate threshold below which artificial oocyte activation with an ionophore is likely to improve outcome.

Based on the findings of Ozil *et al.* (2006), Kashir *et al.* (2010) speculated that 'other infertile patients showing embryo arrest, low embryo quality, and even recurrent implantation failure might also benefit from some form of artificial oocyte activation'. In fact, the present prospective study provides the first evidence that Ca^{2+} ionophore treatment may overcome developmental problems observed in a previous treatment cycle. Intriguingly, this benefit was independent of the stimulation protocol chosen, indicating that simply changing stimulation details may not improve outcome in the bad prognosis patient group showing developmental problems of their embryos. The fact that ~40% of the patients had more than one previous ICSI cycle with developmental incompetence suggests that we were dealing with an intrinsic problem rather than an accidental phenomenon.

Since the positive effect of a ready-to-use ionophore was observed in all three subgroups of patients, it is very likely that complete developmental arrest or delay as well as reduced blastulation are the result of reduced intracellular calcium levels. Obviously, ionophores will ensure sufficient calcium supply for the embryo, even if the Ca^{2+} boost happens immediately after ICSI (as requested by the Ethics Committee). The two patients whose embryos stopped development despite the use of the ionophore might have suffered from senescence (Betts and Madan, 2008).

Any positive effect of ionophore treatment on the rate of mitosis stands and falls with the involvement of calcium ions in the cell cycle. Calcium plays an important role in cell cycle checkpoints from the very beginning (Carroll and Swann, 1992; Tosti, 2006), e.g. spontaneous Ca^{2+} spikes accompany resumption of meiosis in maturing oocytes (Deng *et al.*, 1998; Goud *et al.*, 1999). On the other hand, those gametes shown to be arrested in prophase I are often characterized by the absence of Ca^{2+} channels (Lee *et al.*, 2004). Theoretically, any arrest at later stages, such as metaphase I, could be circumvented by using artificial triggers such as Ca^{2+} ionophores, although no functioning spindle apparatus has yet been observed (Heindryckx *et al.*, 2011).

It goes without saying that calcium initially drives mitotic division (Swanson *et al.*, 1997; Berridge *et al.*, 1998); however, the identification and localization of calcium-binding proteins within human endoplasmic reticulum and IP_3-receptors found to be associated with Ca^{2+} release in human embryos further emphasize that Ca^{2+} also plays a critical role in subsequent cleavages (Goud *et al.*, 1999; Balakier *et al.*, 2002). In contrast, a physiological (Sousa *et al.*, 1996) or artificial (Wong *et al.*, 2005) lack of calcium leads to embryo arrest or cleavage anomalies.

To summarize, this is the first report in humans that developmental incompetence of embryos is an additional indication for ionophore treatment. Apart from IMSI (intracytoplasmic morphologically selected sperm injection) (Knez *et al.*, 2012), the present approach is exclusive in overcoming cleavage arrest. However, we are well aware that a study on sibling oocytes might have demonstrated the actual influence of ionophores on embryo growth much better but our Ethics Committee requested full reliance on a valid method (which was ionophore treatment immediately after ICSI on all oocytes).

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Authors' roles

T.E.: study conception and design, data interpretation; P.O.: revision and final approval of the article; M.W.: acquisition of data, revision of the article; P.S.: drafting and revision of the article; R.B.M.: interpretation of data, revision of the article; U.S.: acquisition of data, revision of the article; S.B.-V.: acquisition of data, revision of the article; I.G.: acquisition of data, revision of the article; A.E.H.: involved in study design, analysis of data; O.S.: critical revision and final approval of the article, acquisition and interpretation of data

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Conflict of interest

T.E. reports fees from Gynemed, outside the submitted work. All co-authors have no interest to declare.

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