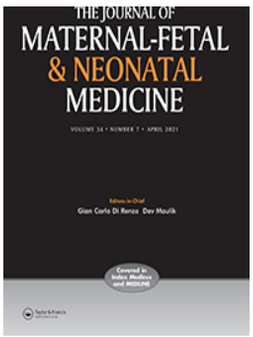

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ORIGINAL ARTICLE

Comparative study between two techniques for artificial shrinkage of blastocysts prior to vitrification: LASER pulse versus micro-needle technique in increasing chemical, clinical pregnancy and live birth rates after ICSI, a randomized controlled trial

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ABSTRACT

Cryopreservation is one of the most important strategies in reproductive medicine now with well-established fertility preservation treatments, successful pregnancy rates.

Purpose: This work investigates ICSI outcome between LASER Artificial Shrinkage (LAS) and Micro-Needle Artificial Shrinkage (MNAS) before vitrification.

Patients and methods: Four hundred and nine women were included in the study; which were randomly divided into two groups according to the technique used for artificial shrinkage step of the blastocyst before vitrification: in the first group, Laser beam was used while in the second group the micro-needle was used. Ovarian stimulation was done before the ICSI procedure either by long, short or antagonist protocol.

Results: The statistical analysis of our study revealed that there was no statistically significant difference between the two groups regarding age, number of cases, AMH, Basal FSH, BMI, male factor, usage ovarian stimulation protocol, high quality blastocysts, the mean number of transferred embryos. While, there was a statistically significant difference between two groups after thawing with p-value $< .001$ in favor of the LAS method regarding the morphology of originally high quality blastocysts, blastocysts healthiness (not degenerated), pregnancy rate, the implantation rates.

Conclusion: LASER artificial shrinkage of human blastocysts is a promising technology that could be implemented on a wider basis to improve ART practice, as our study revealed that the usage of LASER pulse for artificial shrinkage of blastocysts before vitrification has better implantation rate as well as better chemical and clinical pregnancy rate in comparison to the usage of micro-needle artificial shrinkage of blastocysts before vitrification. There is a statistically significant difference regarding live birth rate being more in the LASER group as compared to needle group, also the number of twins ether identical or non-identical are larger in laser group than in needle group but with no statistically significant difference.

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Vitrification; artificial shrinkage; embryo viability; blastocyst

Introduction

Cryopreservation is one of the most important strategies in reproductive medicine now with well-established fertility preservation treatments, and proven successful pregnancy rates [1]. Cryopreservation of embryos encourages the IVF specialists to limit the number of transferred embryos per cycle and thus can lower multiple pregnancy rates. On the other hand, the chances of the cumulative pregnancy rate in an IVF program can be increased without transferring excessive numbers of embryos [2]. Cryopreservation methods can be either by slow freeze or by vitrification methods, but there has been a major shifting to vitrification recently [3].

Vitrification is a modern cryopreservation technique based on sudden cooling of reproductive cells to a temperature of −196 °C in seconds that produces glass-like solidification of a solution without crystallization, but by extreme elevation in viscosity during freezing [3,4]. Unlike the slow freezing method, vitrification has better

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results in the complete elimination of ice crystal inside the cells as well as in the surrounding solution [5,6]. Also, it has a significantly higher post-thawing survival rate in comparison to the slow freezing method [7–9].

Blastocoel in expanding blastocysts may be associated with poorer survival due to potential ice crystal formation. Complete elimination of blastocoel before vitrification can markedly improve outcomes during the preservation of blastocysts [4,10]. Blastocysts can be shrunken artificially by piercing the trophoderm either mechanically by using a micro-needle or by directed a LASER pulse which has been used recently, as an alternative to mechanical collapse with a micro-needle [11,12]. Fast and high re-expansion of the blastocoel can be a strong predictor of the high developmental potential of the blastocysts, and clinical pregnancy outcome [13]. Another advantage of using LASER for artificial shrinkage blastocyst is that it has a faster and higher re-expansion rate compared to artificial shrinkage using a needle [14]. So, this may be a good explanation for the high implantation rate of post-warmed blastocyst after artificial shrinkage by LASER. Also, LASER artificial shrinkage is a time-saving procedure [3]. Also, opening in the zona pellucida created with LASER facilitates and accelerates the hatching process; therefore this process began earlier in artificially collapsed blastocysts [15]. It can be speculated that the single LASER pulse can create heat causing cellular damage of the trophectoderm enough to open a hole in the trophoderm, but less damage in comparison to that caused by the wide tip of the needle [3,16]. Also, collapsing the blastocoel by needle, unlike LASER pulse, requires preparation before the procedure, such as setting the needle, holding the blastocyst at the manipulation stage; while for LASER pulse, its only need is to position the junction of the trophectoderm cells [12]. The simplicity of the LASER technique may make it more advantageous and efficient in an IVF clinical setting [14].

Aim

This work investigates ICSI outcome between LASER Artificial Shrinkage (LAS) and Micro-Needle Artificial Shrinkage (MNAS) before vitrification.

Patients and methods

Four hundred and nine women coming for ICSI at Adam International Hospital were included in the study; which were randomly divided into two groups according to the technique used for artificial shrinkage step of the blastocyst before vitrification: in the first group, Laser beam was used while in the second group the micro-needle was used. Ovarian stimulation was done before the ICSI procedure either by long, short or antagonist protocol.

Blastocysts transfer programs had been performed on cases consented to undergo cryoleaf vitrification protocol to cryopreserve their day 5 blastocysts obtained 5 days after the process of oocyte retrieval. This randomized controlled research trial was performed in Adam Infertility Hospital incorporated with the National Institute of LASER Enhanced Sciences (NILES). Local institutional review board approval was taken before starting the study.

Only cases that had expanded and/or hatching blastocysts had been vitrified on day 5 in the same cryoleaf and have been warmed subsequently for transfer.

Four hundred and nine women were recruited for the randomized controlled trial, their age was ranging from 20 to 42 years old. These 409 cases were categorized into 2 research categories according to the technology used for artificial shrinkage of the blastocyst before performing vitrification: in the first research group, blastocysts had been shrunk using LASER technology while in the second research group the micro-needle technique had been used for artificial shrinkage of the blastocysts.

Ovarian stimulation before the ICSI procedure was done using either long, micro-flare, or antagonist protocol. Ovum Pick-up (OPU) was performed at 35–36 h post HCG trigger. The oocyte cumulus complexes (OCC's) had been collected under vaginal ultrasound guidance. Denudation was done at 38 h post HCG in two steps, chemical then mechanical. Semen samples sperm had been prepared using a gradient separation technique. ICSI had been started one hour after denudation. Fertilization had been checked 16–18 h post-injection. Only fertilized oocytes had been moved to a new culture dish with fresh culture medium (Quinn's Advantage Medium) under oil prepared 12 h before.

Excess embryos were vitrified using Irvine solutions and the McGill Cryo Leaf (Origio), and the center has set protocols for how many embryos transferred according to patient age and previous history. Our rules are transferring 2 good blastocysts (Day 5) to young women ($\leq$39 years old), without a previous history of repeated implantation failures.

Artificial shrinkage of expanded blastocysts was done by one of two procedures

Needle shrinkage

The expanded or hatching blastocyst was held with the flat side of a 29-gauge needle with the inner cell

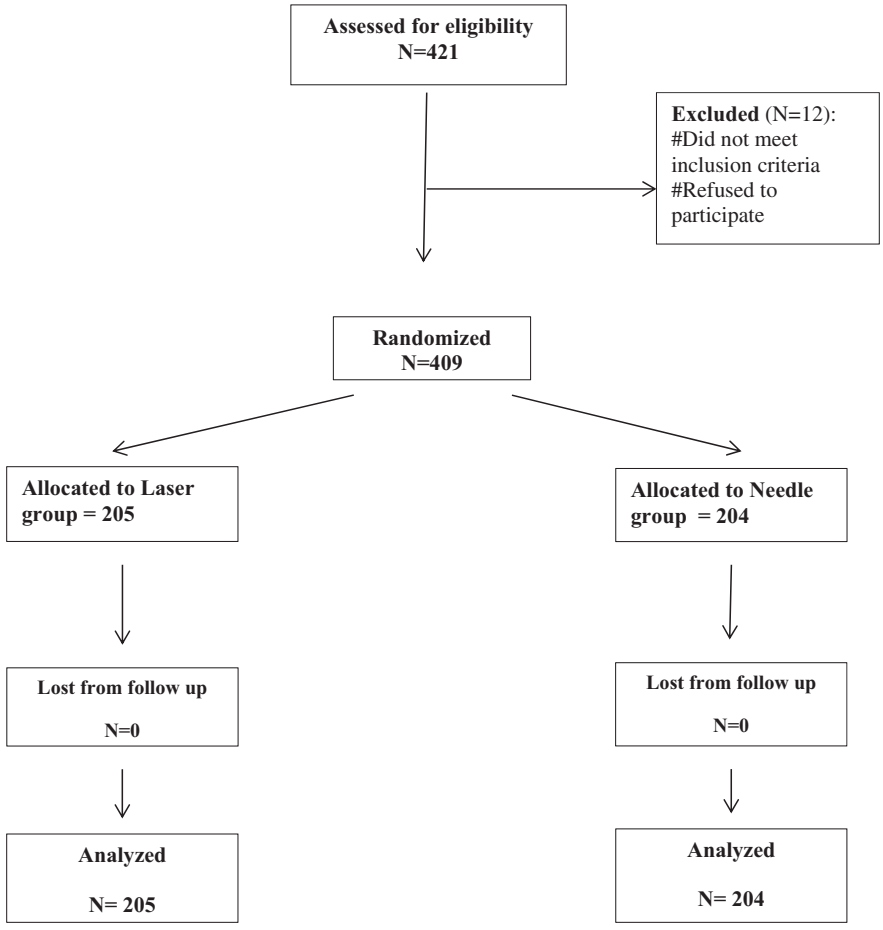

Figure 1. CONSORT diagram.

mass (ICM) at the 12 or 6 o'clock position, a microneedle was pushed through the trophectoderm cells into the blastocoel cavity until the cavity shrank. After complete shrinkage of the blastocoel, the blastocysts were vitrified.

LASER shrinkage

LASER shrinkage was performed using a LASER diode (Saturn diode LASER system) with 1480 nm wavelength operating through an objective lens coupled to an Olympus IX 70 inverted microscope. The ICM was placed away from the targeted point of the LASER pulse. A single LASER pulse (300 μs) was delivered to the outer periphery of the trophectoderm at the junction between the cells. It was sufficient to induce complete shrinkage within 10–15 min. After complete shrinkage of the blastocoel, the blastocysts were ready for vitrification.

Blastocyst transfer and assessment of pregnancy

On the morning of embryo transfer (ET), we warmed the embryos using the Irvine medium for the blastocysts. After warming, we cultured the shrunk blastocysts in BM for 2–3 h to re-expand and then we selected the good looking expanded blastocysts for ET. We performed assisted hatching for the selected blastocysts before the embryonic transfer. We limited to two blastocysts. Only a few patients received three blastocysts, based on their clinical history (i.e. repeat failures or age $\geq$36 years). Pregnancy was assessed by serum hCG 14 days after administration of progesterone, then implantation has been confirmed by the presence of a gestational sac. Clinical pregnancy has been confirmed by the presence of fetal heart activity.

Additionally, we evaluated the morphology of the embryos before vitrification and after warming. The surviving blastocysts have been scored according to their developmental stage, grading of quality, and degree of re-expansion after thawing, considering regain of at least 70% of blastocoel expansion as a good sign.

Statistics

Base line demographics and clinical differences between the two groups have been conducted using independent student *t*-test. The data have been

Table 1. Compares the female age, FSH, AMH, BMI between the Laser AS and Needle AS groups.

	Laser AS		Needle AS		
	N (range)	Mean ± SD	*N* (range)	Mean ± SD	*p*
Female age (years)	205 (21–44)	29.3 ± 0.32	204 (20–41)	29.23 ± 0.33	.834
FSH (mIU/ml)	205 (2.3–17.4)	6.8 ± 2.2	204 (1.8–13)	6.9 ± 1.7	.614
AMH	205 (0–13.2)	2.9 ± 1.9	204 (1.2–14)	3.4 ± 2.2	.264
BMI	205 (17–47)	27.9 ± 4.7	204 (18–40)	27.7 ± 4.1	.642

Significant less than .05.

Table 2. Compares between LASER AS and Needle AS regarding the protocol of stimulation and the male factor.

	Laser AS		Needle AS		
Protocol of stimulation	(*N* = 205)	%	(*N* = 204)	%	*p*
Long (118)	67	32.7	61	30.0	.594
Fixed antagonist	130	63.4	133	65.0	.757
Micro-flare	8	3.9	10	5.0	.640
Male factor					
Easy male[a] (332)	159	48.0	173	52.0	.072
Difficult male[b] (77)	46	59.7	31	40.3	

[a]Easy male factor = easy ejaculate, obstructive azoospermia, countable sperm cells in non-obstructive azoospermia and Easy Fine Needle Aspiration.
[b]Difficult male factor: difficult ejaculate with too few sperm to count, cryptozooospermia, difficult TESE where multiple samples are harvested to retrieve few sperm cells.

Table 3. Compares between LASER AS and Needle AS in the quality of the blastocyst before and after vitrification, healthiness of the blastocyst after thawing, cancelation of the transfer procedure, and ICSI outcome.

	Laser AS		Needle AS		
	N	%	*N*	%	*p*
High quality blastocyst BEFORE vitrification	555/589	94.2	553/584	94.7	.799
High quality blastocyst AFTER vitrification	418/555	75.3	346/553	62.6	.0001
Healthy blastocyst	527/589	89.5	467/584	80.0	.001
Cancelled embryo transfer	4/205	2.0	6/204	2.9	.543
Positive HCG (chemical pregnancy)	141/201	70.1	113/198	57.1	.007
Clinical pregnancy	123/201	61.2	92/198	46.5	.004
Implantation rate	192/527	36.4	121/504	30.4	.0001

Non-significant, where the significant $p < .05$.

analyzed by Chi-square (χ^2) analysis to establish whether differences in implantation and pregnancy rates will be significant between micro-needle artificial shrinkage and LASER artificial shrinkage groups.

Result

The present study showed that a randomized control study between two groups of patients who underwent embryos cryopreservation using the vitrification method before shrinkage of blastocysts the 1st group using LASER technique for artificial shrinkage while 2nd group using Needle technique for artificial shrinkage.

There was no statistically significant difference between two groups regarding age as the mean age in 1st group was 29.3 ± 4.59 years and in 2nd group was 29.2 ± 4.7 years with *p*-value = .834. There were insignificant differences between basal FSH among the two groups as the mean basal FSH in 1st group was 6.8 ± 2.2 IU/L and in 2nd group was 6.9 ± 1.7 IU/L with *p*-value = .614, As well as, BMI as the mean BMI in LAS group was 27.9 ± 4.7 kg/m^2 and in MNAS group was 27.7 ± 4.1 kg/m^2 with *p*-value = .642 as seen in Table 1.

Also, there was no significant difference between different ovarian stimulatory protocols usage in both groups, as long, fixed antagonist, or micro-flare used in the first group represented (33%, 63%, 4%, respectively) in comparison to the second group which represented (30%, 65%, 5% respectively) (Table 2).

Also, there was no statistically significant difference between the two groups before vitrification regarding a number of cases (as 1st group included 204 women and 2nd group included 205 women), high-quality blastocysts (94.2% and 94.7% respectively) with *p*-value = .799 in the two studied groups.

Also, there is no statistically significant difference between the two groups after thawing regarding the mean number of transferred embryos in which 2.6 ± 0.05 in LAS group and 2.5 ± 0.06 in MNAS group, as well as, cancelation of the transfer procedure due to absent healthy embryos showed no statistically significant difference with *p*-value = .543 in both groups as shown in Table 3.

After thawing, the comparison between two groups showed a highly statistically significant difference between two groups with *p*-value < .001 in favor of the LASER artificial shrinkage method (1st group) regarding the morphology of originally high quality blastocysts (75.3% compared to 62.6%, respectively), as shown in Table 4, as well as blastocysts healthiness (not degenerated) after warming with more healthy blastocysts in LASER AS shrinkage group (1st group) 89.5% than needle AS (2nd group) 80.0% as demonstrated in Table 5.

Moreover, there is a significant difference between two groups in favor of the LASER artificial shrinkage method regarding either both early chemical pregnancy (ß-HCG positive) before ultrasound detection of fetal pole with 70.1% positive in LASER AS group (1st

Table 4. Comparison of both groups regarding the morphology of originally high quality blastocysts after warming.

Artificial shrinkage method * Quality after vitrification cross tabulation							
			Quality after vitrification		Total High quality blastocyst before vitrification	Chi-square	
			High quality	Bad quality		Exact sig. (2-sided)	Exact sig. (1-sided)
Artificial shrinkage method	LASER AS	Count	418	137	555	<.001[a]	.000[a]
		% Within artificial shrinkage method	75.3%	24.7%	100.0%		
	Needle AS	Count	346	207	553		
		% Within artificial shrinkage method	62.6%	37.4%	100.0%		

[a]Highly significant $p < .001$.

Table 5. Comparison between the two groups regarding quality of blastocyst degeneration after warming: artificial shrinkage method * healthiness after warming cross tabulation.

			Healthiness after warming			Chi-square	
			Healthy	Degenerated	Total	Exact sig. (2-sided)	Exact sig. (1-sided)
Artificial shrinkage method	LASER AS	Count	527	62	589		
		% Within artificial shrinkage method	89.5%	10.5%	100.0%	<.001[a]	.000004[a]
	Needle AS	Count	467	117	584		
		% Within artificial shrinkage method	80.0%	20.0%	100.0%		

[a]Highly significant $p < .001$ in favor of the LASER artificial shrinkage method.

group) compared to 57.1% positive in needle AS group (2nd group) with p-value = .007, or in which clinical pregnancy rate was 61.2% in LASER AS (1st group) compared to 46.5% in needle AS group (2nd group) with p-value = .004 (Table 4).

While regarding the implantation rates in our study, revealed that there was a highly statistically significant difference between the two groups in favor of the LASER artificial shrinkage group as the implantation rates in LASER AS (1st group) is 36.4% compared to 24.0% implantation rate in needle AS group (2nd group) with p-value < .001 (Table 4).

Discussion

Artificial shrinkage of blastocysts before vitrification improves implantation, pregnancy, and delivery rates as it decreases the risk of cryo-damages of the blastocysts ultra-structural [17].

Many studies are in harmony with the results of our research in comparing and contrasting survival rates, clinical pregnancy rate, and implantation rate, between LAS and MNAS technique. As our study showed that there were no statistically differences in blastocyst survival rates (98% vs 97.1% respectively, $p = .543$) between both groups, while there was a statistically significant difference between two groups in favor of the LAS regarding clinical pregnancy rate which was 61.2% in LAS compared to 46.5% in MNAS with p-value = .004, as well as the implantation rates that revealed 36.4% compared to 24.0% respectively with p-value < .001.

Many researches reached the same conclusion about the superiority of LAS for blastocysts before vitrification on the outcome after thawing in comparison to untreated (non-collapse) blastocysts. Previous research revealed that the survival rate was significantly higher in the LAS group compared with the control group (97.3 and 74.9%, respectively; $p < .01$), also the clinical pregnancy was significantly higher in LAS group 67.2 vs. 41.1%; respectively; $p < .01$), as well as in the implantation rate (39.1 vs. 24.5% respectively; $p < .01$) [10]. Also another study showed that LAS group had higher statistically survival rate (100% vs 91% respectively), positive β-hCG (66.9% vs 59.7%), clinical pregnancy rate with fetal heart beat (56.4 vs 49.6), even higher quality blastocysts after thawing in comparison with untreated (non-collapse) blastocysts (39% vs 22% respectively; with p-value = .02) [18]. On the other hand, some authors revealed that there were no statistically differences in blastocyst survival rates (95.40% vs 94.05%, $p > .05$) between the LAS and MNAS groups. However, compared with MNAS, LAS improved the warmed clinical pregnancy rate (60.82% vs 54.37%, $p < .05$) [8]. Another research also had no statistically significant difference between the two methods in the rates of blastocyst survival between LAS and MNAS (92.8% vs. 93.0%, respectively), but disagreed with the results of our research in the clinical pregnancy, and implantation rates as it showed no statistically significant difference between the two groups. The clinical pregnancy rates for each group were similar (60.78% vs. 60.36% respectively). Also, implantation rates in the LASER pulse AS group were slightly higher than those in the micro-needle AS group (44.6% vs 41.8% respectively), although

these differences were not statistically significant [16]. Also, there were no statistically significant differences in the survival rate between LAS (100%) and MNAS (97%) [5]. On the other hand, another study revealed no difference at all between the two groups with survival rate 100% (73/73) [19,20].

A little damage in the blastocyst can occur during artificial shrinkage but more importantly, it can improve the viability of the blastocyst after thawing and the pregnancy rate after embryo transfer. The morphology of high quality blastocysts was similar between the two groups before vitrification (94.2% and 94.7% respectively) with *p*-value = .7992, while after thawing, embryos treated with LAS were superior (75.3% compared to 62.6%; 0.001, respectively). Many researchers conducted very similar results in our study. As some authors concluded from their research that good embryo rate showed no difference between the two groups before freezing (74.52% vs. 70.87%; $p = .392$) but showed a significant difference after thawing in favor of the LAS group (75.19% vs. 45.33%; $p < .001$) [16].

Other researchers evaluated the impact of artificial shrinkage by different techniques on blastocysts by observing the rate of re-expansion to 70% of the original volume. One study revealed that Seventy-three blastocysts were shrunk by using a MNAS; 76.7% (56/73) were re-expanded up to at least 70% within 1 h. The remaining blastocysts 23.3% (17/73) were re-expanded within 2 h, while in LAS group 84.9% (62/73) were re-expanded up to at least 70% within 1 h and 15.1% (11/73) were re-expanded within 2 h. There was no degeneration of the shrunken blastocysts [21]. Another research revealed the same conclusion that artificial collapse by either mechanical puncture or LASER pulse and the consequent reduction of internal fluid volume significantly minimized cell damage during vitrification -warming and enhance post-warming re-expansion, as 81% of LAS and 73% of MNAS collapsed blastocysts were fully re-expanded as compared to only 53% of blastocysts that vitrified without reduction of blastocoel ($p < .001$). But they detected a significant increase in blastomeres damage in the control group (13%) in comparison to blastocysts artificial collapsing before to vitrification (LAS 3%, MNAS 5%; $p < .001$) [11,19]. Previous studies recommended proper investigation and preparation of women with secondary infertility in the pre-ICSI work up with previous cesarean scar for better ICSI outcome [22].

The rate of re-expansion of the blastocyst during thawing was one of our criteria in the morphological evaluation, but we evaluated the blastocysts 2 h after thawing. Our results were in line with those researches in that LAS had the best result on the blastocysts morphology after thawing. As well as in healthiness (not degenerated) of retrieved blastocysts after warming either from the total vitrified blastocysts or high-quality blastocysts. As the artificial shrinkage regarding the healthiness of retrieved blastocysts after warming showed highly significant difference between LAS group 89.5% (527/589) and MNAS group 80% (467/584) with *p*-value $< .001$ in favor of the LASER artificial shrinkage method, also we studied the retrieval of high quality blastocysts after warming (from high quality vitrified blastocysts) which revealed also a highly statistically significant difference between both groups (75.3% vs 62.6% respectively) with *p*-value $< .001$ in favor of the LAS method.

Table 6. The number of twins in both groups.

	Twin cases			
	Identical twins	Unidentical twins	Total	*p* Value
LASER AS	7 (20%)	28 (80%)	35	.725
Needle AS	3 (13.6%)	19 (86.4%)	22	

Chi-square tests.

Table 7. The comparison between both groups regarding live birth rate.

	Baby take home			
	Yes	No	Total	*p* Value
LASER AS	103 (51%)	99 (49%)	202	.012
Needle AS	76 (38.4%)	122 (61.6%)	198	

Chi-square tests.

Table 8. The live birth rate (baby take home rate) in both groups.

	Single baby take home rate			
	Single baby	Twins or more	Total	*p* Value
LASER AS	67 (65%)	36 (35%)	103	.525
Needle AS	53 (69.7%)	23 (30.3%)	76	

Chi-square tests.

A recent systematic review and meta analysis in 2020 revealed that artificial shrinkage before blastocyst vitrification enhances survival and clinical pregnancy rate, but not implantation or live birth rate and more randomized researches are needed to improve the level of evidence and confirm these findings [23].

A recent study showed that endometrialmanipulation could increase pregnancy rate and live birth rate [24]. Moreover, in the current study, there is a statisticaly significant difference regarding the total live birth rate being more in the LASER group as compared to needle group, also the number of single and twins ether identical or non identical are larger in laser group than in needle group but with no statistically significant difference as shown in Tables 6–8.

Conclusion

LASER artificial shrinkage of human blastocysts is a promising technology that could be implemented on a wider basis to improve ART practice, as our study revealed that the usage of LASER pulse for artificial shrinkage of blastocysts before vitrification is more simple technique with less damage to the embryos with better implantation rate as well as better chemical and clinical pregnancy rate in comparison to the usage of micro-needle artificial shrinkage of blastocysts before vitrification . However further and more detailed issues should be involved in future research to reveal possible influences on the perinatal and postnatal progress of children born after the use of either method.

There is a statistically significant difference regarding live birth rate being more in the LASER group as compared to needle group, also the number of twins ether identical or nonidentical are larger in laser group than in needle group but with no statistically significant difference.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This randomized controlled research was approved by Local institutional review board.

Informed consent

Informed consent was obtained from all the individual participants included in the study.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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