

Gold nanoparticles improve the embryonic developmental competency of artificially activated mouse oocytes

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Abstract

Currently, artificial oocyte activation has attracted wide attention in assisted reproduction due to extensive range of applications, particularly in somatic cell nuclear transfer and deriving pluripotent stem cell lines and it is the unique model to determine the role of paternal genome. Numbers of artificial activating agents have been used extensively to induce the oocytes activation; however, embryos developmental competency of artificially activated oocytes is still very low. In the present study, we determined the functional impact of strontium chloride supplementation with gold nanoparticles (AuNPs) in artificial oocytes activation and subsequent embryonic development. Oocytes were activated artificially in the culture medium containing 250 nM AuNPs with constant concentration of strontium chloride 10.00 mM. We found that adding 250 nM AuNPs with constant concentration of strontium chloride (10.00 mM for 3 hr) in culture medium improves the proportion of embryos reaching to the morula and blastocyst stages from 61.00% and 42.00% (controls) to 75.00% and 58.00% (250 nM AuNPs), respectively. In addition, foster mothers receiving AuNPs-treated embryos showed more implantation percentage and pregnancy rate relative to females received control embryos. Finally, embryos treated with 250 nM AuNPs concentration showed no toxic effect in term of blastocyst development. Collectively, our findings suggest the potential role of AuNPs in early embryonic development for mouse oocytes activated artificially and provide new insights in the field of animal biotechnology and assisted reproduction in humans.

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Introduction

Recently fertilized oocytes are the tools for embryonic stem cell research. The totipotency and developmental potential of stem cells originated by parthenogenetic activation resemble with the stem cells originated from natural fertilization.¹ The achievement in the technology of nuclear transfer for cloning is based on efficient activation of oocytes.² Currently, parthenogenetic activation technique has become the prime consideration in reproductive biology with wide range of applications in somatic cell nuclear transfer, somatic cell cloning, assisted reproduction and deriving pluripotent stem cell lines. Activation of mature oocytes is an essential step in establishment of successful embryo reconstruction by

nuclear transplantation.³ Artificial activation, known as parthenogenesis, is not a single step process in the field of reproductive biology; however, it involves series of events starting with succession from oocyte activation until blastocyst formation. Calcium (Ca²⁺) oscillation is the most notable event occurred during oocyte activation.⁴ During this process, influx of Ca²⁺ increases⁵ through the stimulation of inositol triphosphate receptor (IP₃)⁶ causing protein kinase C activation and ultimately reduction in maturation promoting factors (MPFs).⁷ Reduction in MPFs level is associated with meiotic arrest resumption as occurs in natural process of sperm-egg fusion.⁵

Physical and chemical methods are the renowned methods used for oocytes activation. Strontium chloride (SrCl₂) is one of the foremost chemical activating agent

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used for mouse oocytes activation; however, the embryo development reaching to the blastocyst stage is still compromised.^{8,9} Thus, it is important to develop some strategies which could improve the embryonic development of artificial activated oocytes. Nanotechnologies had shown propitious potential in different sectors of everyday life. Unique properties and small size of nano-materials have been used in scientific and medical fields including pharmacology, medicine and bioinformatics.¹⁰ Metal nanoparticles had displayed eccentric electronic, magnetic, catalytic and optical properties attributing unique functional activity compared to bulk metals.

Gold nanoparticles (AuNPs) as one of the most precious particles have shown potential applications in research and clinics. Small size with versatile shape has determined its functional activity during transportation of molecules into the cell.¹¹ Size and shape of the particles were considered as essential factors being correlated with their cellular interactions.¹² The gold would not be considered as a catalyst few years ago due to its noble properties. Previously, it was suggested that AuNPs act as catalyst by aerobic oxidation of carbon monoxide. The AuNPs are accompanied with properties required for catalysis including low coordination, small size and interface with scaffold.^{13,14} The catalytic activity of AuNPs also has been determined in free solution state.¹⁵ Previous reports have demonstrated that AuNPs with 1-100 nm size range are associated with increased catalytic activity.¹⁶ Previously, the functional impacts of AuNPs were determined in embryos and data suggested the biocompatibility of these particles with embryos.¹⁷

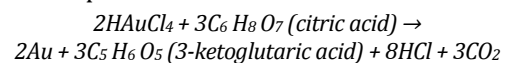
The functional activity of SrCl₂ for mouse oocytes activation is quite acceptable; however, the rate of embryos reaching to the blastocyst stage is very low. In this study, we demonstrated that adding 250 nM AuNPs with constant concentration of SrCl₂ (10.00 mM) in culture medium improves the proportion of blastocyst formation, implantation rate and pregnancy outcome with no detrimental effect on embryos development.

Materials and Methods

Oocytes and embryos collection. For the collection of oocytes and zygotes, BALB/c female mice with approximate weight of 21.00 g (7 - 9 weeks) were super-stimulated with 5.00 IU pregnant mare serum gonadotropin (Intervet, Boxmeer, Netherlands) followed by 5.00 IU of human chorionic gonadotropin (hCG; Intervet) after 48 hr interval, intra-peritoneally. Females were sacrificed and oocytes were collected after 16 hr of hCG administration. For collection of zygotes, female mice were exposed to male mice with proven fertility and formation of vaginal plug was confirmed. The metaphase II (MII) stage oocytes and zygotes were collected by treating cumulus oophorus with 300 µg mL⁻¹ hyaluronidase (Sigma-Aldrich, St. Louis, USA) in M2 media (Sigma-

Aldrich) with gentle pipetting for 1 min. The oocytes/zygotes were maintained at 37.00°C with 5.00% CO₂ prior to activation. All the experimental protocol was performed in accordance with the guideline of Ethical Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan, with ethical number of No/DR: ORIC 128.

Preparation of AuNPs (sodium citrate reduction method). Chloroauric acid (HAuCl₄) and trisodium citrate solutions were prepared. Fifteen mL of 0.0001M HAuCl₄ solution was heated at 65.00 °C until solution began to boil. A volume of 0.10 mL of 0.04 M solution of trisodium citrate was added. Both solutions were heated for 15 min until the color change was evident. After removing from the heating element, the solution was stirred and made cold at the room temperature. Chemical reaction is as follows:



The AuNPs size distribution depends on the concentration of gold salt, trisodium citrate, temperature and mixing rate. The UV-visible spectra of AuNPs were performed falling in absorption spectra peak at a wave-length of 521 nm indicating the particles size of 20.00 - 50.00 nm; while, 524 nm indicates particles size of 50.00 - 160 nm.

Oocytes activation with AuNPs in SrCl₂ solution. To investigate the effect of AuNPs on artificial activation, oocytes were exposed to different concentrations of AuNPs (0.00, 50.00, 250, 450 and 650 nM) with constant concentration of SrCl₂ (10.00 mM for 3 hr) in the Ca⁺²-free Modified Chatot, Ziomek, and Bavister (CZB) media supplemented with cytochalasin B (5.00 µg mL⁻¹). After 3 hr of activation in SrCl₂ supplemented with AuNPs in the culture medium, oocytes were washed and used for further culturing experiment. After activation, oocytes were incubated in M16 culture media (Sigma-Aldrich) at 37.00°C with 5.00% CO₂ until evaluation of activation and further developmental potential.

Embryo transfer. Compact morulae and blastocysts obtained from AuNPs and control groups activation were transferred to the uterine horn of pseudo-pregnant females (anesthesia of these females was guided by Jamal *et al.*¹⁸ Female mice were sacrificed at 5.50 days post conception (DPC) and uterine horn was dissected immediately. The embryos were separated tearing the membranes using fine forceps. Number of implanted embryos was counted as an indicative of implantation percentage. Pregnancy and implantation percentages were determined at 5.50 DPC.

Toxicity of AuNPs. To elucidate the toxic effect of AuNPs on embryos, zygotes were exposed to different concentrations of AuNPs (0.00, 250, 450 and 650 nM) in potassium simplex optimization medium (KSOM) (Sigma) for 3 hr. After several washings, zygotes were incubated in KSOM without AuNPs at 37.00°C with 5.00% CO₂ for embryonic development examination.

Statistical analysis. The data were analyzed using statistical software SAS Enterprise Guide (version 4.2; SAS Institute Inc., Cary, USA) and statistical significance was set at $p < 0.05$. Binary Logistic regression test was used for cleavage as well as morula and blastocyst development analyses.

Results

To test the role of AuNPs in oocytes activation and embryonic development, the MII-stage oocytes were retrieved and cultured in activation media containing 10.00 mM SrCl₂ supplemented with different concentrations of AuNPs after hormonal stimulation. No significant difference was observed in oocytes activation; however, 250 nM concentration of AuNPs showed significant improvement in proportion of zygotes reaching to morula and blastocyst stages compared to control embryos. Most embryos in control group arrested at different embryonic stages before reaching to the blastocyst stage compared to the treatment group (Fig. 1). In addition, the percentage of dead oocytes was also monitored by observing number of dead oocytes with reference to total number of oocytes used for activation and we found that the numbers of dead oocytes were very low at optimum concentration of AuNPs. However, percentage of dead oocytes was increased at higher concentration of AuNPs (Table 1).

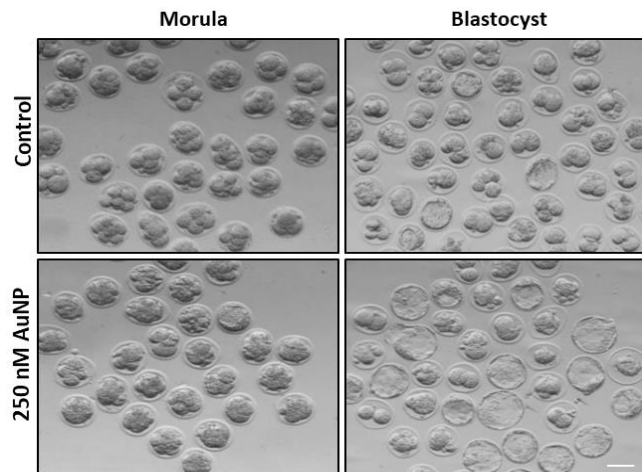


Fig. 1. Morphology of activated embryos using strontium chloride with and without AuNPs (Scale bar: 100 μm).

Table 1. Effect of gold nanoparticles (AuNPs) on parthenogenetic activation of mouse oocytes.

AuNPs (nM)	No.	Dead oocytes		2-cell (24 hr)		Morula (72 hr)		Blastocyst (96 hr)	
		%	Odd ratio	%	Odd ratio	%	Odd ratio	%	Odd ratio
0.00	123	15.00	-	88.00	-	61.00	-	42.00	-
50.00	100	14.00	0.95	93.00	1.80	65.00	1.19	45.00	1.10
250	130	15.00	1.06	90.00	1.20	75.00	1.90*	58.00	1.80*
450	80	29.00	2.50*	86.00	0.84	48.00	0.59	15.00	0.23*
650	70	44.00	4.63*	87.00	0.96	41.00	0.45	90.00	0.14*

Oocytes activation was achieved by strontium chloride (10.00 mM) with or without AuNPs for 3 hr exposure. Cleavage, morula and blastocyst rates were evaluated 24, 72 and 96 hr after treatment. Binary Logistic regression was used as a statistical tool. Asterisk indicates significant difference at $p < 0.05$.

Thus, these results suggest that 250 nM concentration of AuNPs with 10.00 mM SrCl₂ is suitable for improving the early embryos development.

For further verification of the developmental fate of AuNPs-treated embryos *in vivo*, control and 250 nM AuNPs-treated blastocyst-stage embryos were used for embryo transfer experiment. Foster mother transferred embryos treated with AuNPs showed higher pregnancy rate and improved implantation percentage compared to the control (Fig. 2, Table 2).

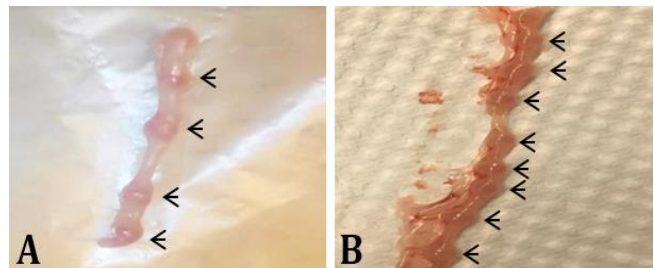


Fig. 2. Morphology of uteri of recipient females after embryo transfer **A)** control and **B)** AuNPs treated-groups. Fifteen embryos were transferred per female. Less than 5 recipient females were used in each group. Arrow heads indicate the sites of implantation.

Table 2. Effect of gold nanoparticles (AuNPs) on *in vivo* developmental potential of parthenogenetic activated mouse blastocysts.

AuNPs (nM)	Blastocysts transferred	Recipients 2.50 DPC	Pregnant recipients (%)	Implantations 5.50 DPC (%)
0.00	90	6	2/6 (33.30%)	34.00
250	90	6	3/6 (50.00%)	48.00

Blastocyst was transferred to the pseudo-pregnant females treated with or without AuNPs into the uteri. Fifteen blastocysts were transferred per female. DPC: Days post conception.

To evaluate the toxic effect of selected concentration of AuNPs on embryos development, wild type (WT) females were mated with WT male and zygotes were collected after successful mating. The zygotes were cultured in medium with 250 nM AuNPs concentration. No toxic effect of 250 nM AuNPs concentration was observed in term of embryonic development compared to the un-treated embryos (Table 3, Fig. 3).

These results imply that 250 nM AuNPs concentration has no detrimental effect on mouse embryos development.

Table 3. Toxic impact of gold nanoparticles (AuNPs) on embryonic development.

AuNPs (nM)	No.*	2-cell (24 hr)		Morula (72 hr)		Blastocyst (96 hr)	
		%	Odd ratio	%	Odd ratio	%	Odd ratio
0.00	62	92.00	-	84.00	-	74.00	-
250	63	86.00	0.52	81.00	0.82	70.00	0.84
450	60	83.00	0.43	80.00	0.75	70.00	0.83
650	58	81.00	0.37	72.00	0.49	60.00	0.52

Mouse zygotes (24 hr post-hCG) were exposed to AuNPs for 3 hr. Two-cell, morula and blastocyst rates were evaluated 24, 72 and 96 hr after treatment, respectively.

* indicates the total number of zygotes. Binary Logistic regression was used as a statistical tool.

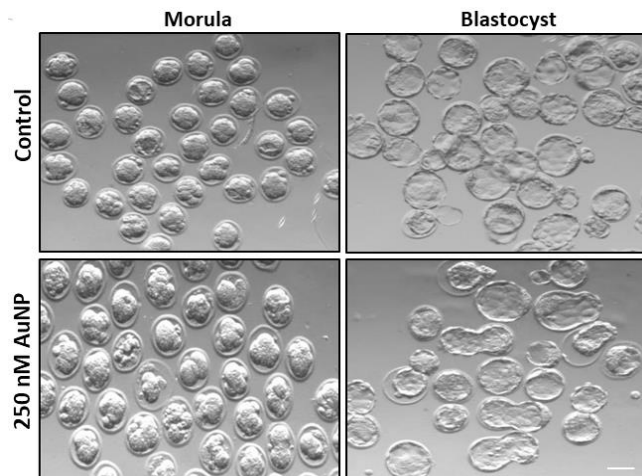


Fig. 3. Morphology of zygotes derived from wild type female and treated with 250 nM AuNPs concentration in cultured media (Scale bar: 100 μ m).

Discussion

Artificial activation of oocytes has been reported in number of species including mouse,¹⁹ rat,²⁰ rabbit,²¹ cow,²² pig²³ and human²⁴ using different activating agents. Previous studies had determined the optimum activation percentage of mouse oocytes with different activating agents; however, the embryonic development is still not up to the optimum level.^{9,25,26} In the present study, we observed that 250 nM AuNPs concentration in activation medium (SrCl₂) is optimum to improve the blastocyst proportion. We conducted different trials to optimize the concentration of AuNPs and 250 nM AuNPs ensure the best concentration regarding embryonic development. Our data is comparable with previous reports regarding oocytes activation potential which was examined as 53.00%;^{26,27} however, the increased blastocyst percentage was observed in our findings with AuNPs supplementation in activation medium compared to previous reports used same SrCl₂ concentration for same duration.^{9,22,26}

The key factor for meiotic resumption is Ca²⁺ oscillations. During artificial activation, Ca²⁺ oscillations occur through stimulation of IP₃,⁶ which in turn increases the cytosolic Ca²⁺.⁵ Addition of AuNPs in activation media might be mediated the effect of SrCl₂ by increasing Ca²⁺ oscillations and membrane permeation resulting in higher

Ca²⁺ influx inside the cytosol leading to resumption of meiosis. It was determined in different studies that Ca²⁺ transient promotes the embryonic development, implantation and subsequent post-natal development.²⁸ Previous reports have shown that nanoparticles are widely used in drug delivery in cancer therapy and promote the drug penetration.^{29,30} Previously, it was reported that peptide nanoparticles have been used efficiently for gene knockdown in mouse oocytes in siRNA transfection system.³¹ In recent study, protein inhibition for mouse oocytes was also confirmed using peptide nanoparticles with target drug in the culture medium.³² These findings suggest that nanoparticles might be useful for mediating the penetration of drug into the oocytes when added in the culture medium. Our data also suggest that AuNPs might be beneficial to facilitate the penetration of SrCl₂ into the oocytes in the culture medium. These reports have consented the functional activity of AuNPs to support the SrCl₂ in culture medium. The potential functional impacts of AuNPs as catalysts in different biological processes were also determined irrespective of promising effect in drug delivery.³³ Thus, addition of AuNPs in activation media might be essential to improve the early embryonic development by supporting SrCl₂ in the medium or acting as potential catalysts; however, the exact mechanism still needs to be investigated.

Furthermore, the developmental fate of AuNPs-treated embryos was determined by *in vivo* study through embryo transfer experiment which showed higher pregnancy rate and improved implantation percentage in treatment group compared to the control. For further investigation of the toxic effect of AuNPs, zygotes were exposed to different concentrations of AuNPs and results indicated no toxic effect on developing embryos. Previous studies on zebra fish and chicken derived embryos have shown non-toxic effects of AuNPs on the embryonic development supporting our findings.^{17,34,35}

Currently, parthenogenetic process has attracted great attention consistently due to its wide range of applications in assisted reproduction for the fertility treatment and research work including round spermatid injections, stem cell research and somatic cell nuclear transfer.³⁶ It might be useful in human fertility process *in vitro* fertilization clinics during intra-cytoplasmic sperm injection (ICSI) protocol to activate the oocytes. Recent report has

indicated the application of artificial activation in assisted reproduction technology especially in ICSI for the selected patients with fertilization and embryonic developmental problems.³⁷

In conclusion, the present study provides compelling evidence that 250 nM AuNPs concentration is suitable to improve the blastocyst proportion when supplemented in activation media with minimal detrimental effect. However, further studies are required to investigate the mechanism of Ca²⁺ rise and pattern of Ca²⁺ oscillation in embryos treated with AuNPs.

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Conflict of interest

There is no conflict of interest to declare.

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