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研究課題名（和文）Production of 2-D nanocarbons with different bioaffinities as ROS quenching agents to improve mammalian oocyte cryopreservation outcomes

研究課題名（英文）Production of 2-D nanocarbons with different bioaffinities as ROS quenching agents to improve mammalian oocyte cryopreservation outcomes

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研究成果の概要（和文）：女性の生殖細胞の凍結保存技術を向上させるために新しい解決策が必要です。酸化グラフェン（GO）が卵母細胞の凍結中にどのように保護するか評価しました。まず、GOが体外培養環境で卵母細胞に有害性を示すか評価しました。安全性を確認した後、凍結中の適用性を評価しました。凍結時に直接使用すると、GOと細胞の強い接着により細胞死亡率が上昇しました。回復時にのみ使用すると、細胞の生存率がわずかに向上する効果がありましたが、一貫性に欠けます。GOの表面修飾がさらに必要とされ、卵母細胞の凍結保存における効果的な利用に向けた研究が求められます。

研究成果の学術的意義や社会的意義
新しい材料の発見と実用的な応用拡大は技術の進展に不可欠です。特に脂質が豊富な卵母細胞は低温ダメージに非常に敏感であり、その凍結成功を改善する進展はほとんどありませんでした。これは生物多様性の保護と動物の繁殖・遺伝学産業の向上に深刻な課題を投げかけます。
この研究の結果は、酸化グラフェン（GO）などの2次元材料が凍結保存技術の限界を克服する基盤としての潜在性を示しています。純粋なGOは卵母細胞の凍結保存に成功しませんでしたが、そのクライオプロテクタントとしての特性を他の分子で修飾する可能性は無限であり、この分野での研究を進める努力が必要です。

研究成果の概要（英文）：It is necessary to find new solutions to improve cryopreservation technologies for female gametes. For this, we evaluated the capacity of graphene oxide (GO) to protect oocytes during cryopreservation.
For this, we evaluated whether GO exhibited any toxicity or presented any apparent negative effects to oocytes in an in vitro culture setting. After confirming the safety of GO, we proceeded to evaluate its applicability during cryopreservation. When used during the freezing stage, the oocytes presented a higher degree of mortality than those in the control ones, due to mechanical damage caused by a strong adhesion between the cells and GO. In contrast, when used just during the recovery stage, GO exhibited a slightly positive effect to maintain cell viability, but the results were not concluding. With this study, we now understand that further surface modifications are necessary to improve the capacity of GO to be used effectively during oocyte cryopreservation.

研究分野：0501

キーワード：graphene oxide　reduced graphene oxide　oocyte　vitrification　2D materials

１．研究開始当初の背景

Cryopreservation is an assisted reproductive technology (ART) which consists in freezing gametes and embryos, thus allowing the long-term storage of germplasm. The process of freezing-and thawing living cells causes serious damage because of the formation of ice crystals and the excessive production of reactive oxygen species (ROS), which damages the lipidic membranes of cells and organelles, causing caspase activation, which will trigger apoptosis and cell death.

The use of carbon nanomaterials is gaining importance because their properties make them to be potentially applicable in many biological fields. Some of their advantages are: (i) low cost and toxicity, (ii) surface chemistry, such as oxidation levels, easily modifiable, (iii) easy modification by polymer grafting, (iv) controlled ROS production/quenching capacity, etc. ***Graphene*** *is a 2-D carbon material known for its great electric and thermal conductivity, high mechanical strength, and extensive surface area.* ***When treated with strong oxidants****, the surface of graphene* ***acquires several oxygen groups such as carboxyl, hydroxyl, and epoxy, and is then known as graphene oxide (GO)****. GO differs from the former, because of its lower conductivity and surface area, as well as, higher hydrophilicity depending to its oxidation level.*

According to the size, shape, surface charge and functional groups, its capacity to produce/quench ROS will vary, making GO a potential candidate for biological applications such as antimicrobial agents, drug delivery, cancer therapy, etc. Among nanocarbons, fullerenes and nanotubes have already been demonstrated to act as ROS donors or quenchers, specially toward $HO^{\cdot}$, $^1O_2$ *and* $O_2^{\cdot-}$*. This versatility is one of the reasons why they are considered good candidates as oxidative stress inhibitors. In the case of* ***2-D carbons, such as graphene and GOs, further investigations including mechanistic studies are required to determine their applicability as efficient ROS quenchers****.*

２．研究の目的

To avoid the loss of irreplaceable genetic material, I have ***proposed the use of graphene-derived nanomaterials with different oxygen contents surface chemistry as ROS quenching agents to be added during or after the cryopreservation process to protect gametes from oxidative stress damage****. Hence, the production of high-quality oocytes and the success increase of ART treatments involving cryopreservation is expected through the following investigations:*

(1) Development of non-toxic highly-stable nanocarbons with finely-tuned ROS quenching capacity.

(2) Evaluation of cell toxicity and analysis of the capacity of GO with different oxygen contents to protect oocytes from oxidative stress during and after cryopreservation.

３．研究の方法

The project was planned to be executed into two well defined stages:
(1) (FY2022): Production of graphene-derived nanomaterials and analysis of their structure and ROS quenching activity.
During this stage, the production of 2-D nanocarbons with different oxygen contents and their biocompatibility optimization by functionalization with several oxygen and organic groups while avoiding cell toxicity and promoting ROS quenching activity is planned. The size and structure of the nanocarbons will be assessed with XPS, AFM and SEM analyses. For the assessment of the ROS production/quenching activity of the functionalized nanocarbons ESR analysis by spin trapping is to be performed.

(2) (FY2023): Analysis of nanocarbon toxicity and optimal concentrations for adequate ROS quenching in oocyte cryopreservation.

During the second part of the project, the toxicity of the nanocarbons will be assessed by oocytes co-incubating to elucidate the range of concentrations that are able to maintain or promote cell survival. After evaluating the working ranges of the materials, their effects during the cryopreservation process will be further analyzed as well as examining if the materials are able to prevent oxidative stress by the quantification of the expression levels of several intracellular markers (superoxide dismutase 1, peroxiredoxin 3, thioredoxin 2, etc.) by RT-PCR.

４．研究成果

(1) Production of graphene-derived nanomaterials and analysis of their structure and ROS quenching activity.

Graphene oxide was synthesized by a modified Hummers method according to a well-established protocol ((Morimoto N. et al. Sci. Rep. 2016, 6, 21715). To modify the levels of surface oxidation to produce reduced graphene oxide (rGO), the use of a thermal method was selected to avoid the presence of harsh chemicals such as hydrazine that could negatively affect cell viability. For this, GO was reduced at 80° C through the span of different time periods (0 h, 12 h, 24 h, 48 h, 72 h, 168 h and 336 h). After that, the resulting materials were characterized via AFM, SEM, TGA, XPS, UV-Vis and the presence of active groups on the surface was evaluated by ESR. Even if the ***results indicated that the higher the degree of reduction of the rGOs, the higher their ESR activity*** *(Fig 1),* ***due to their poor solubility in water*** *(Fig 2), rGOs were not used for the preliminary in vitro viability studies.*

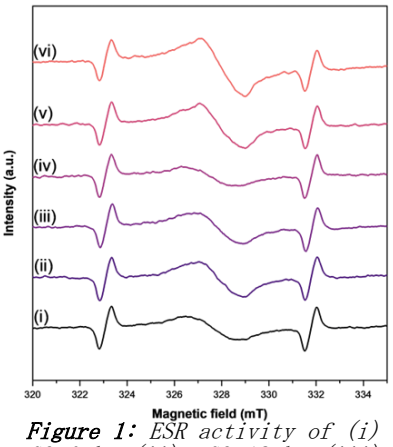

Figure 1: *ESR activity of (i) GO 0 h, (ii) rGO 12 h, (iii) rGO 24 h, (iv) rGO 72 h, (v) rGO 168 h and (vi) rGO 336 h*

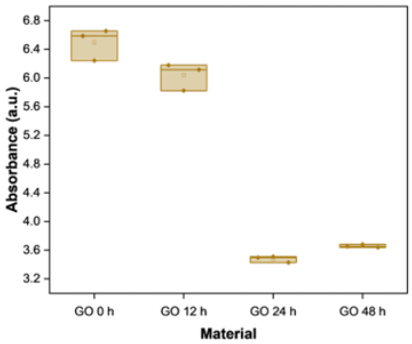

Figure 2: *UV-Vis analysis of GO and rGO at a dilution (1:500) after 15 minutes of settling. rGO does not disperse well in water as demonstrated by the significantly lower absorbance presented, especially after 24 h of thermal treatment.*

(2) Analysis of nanocarbon toxicity and safety in an oocyte in vitro culture setting.

When GO was used during routine in vitro maturation of porcine oocytes (Ferré P. et al. Theriogenology 2016, 86, 1705), its presence slightly improved oocyte viability at a concentration of 100 μg/mL although not significantly different of the controls in the absence of other antioxidant molecules. Similarly, the addition of GO did not affect nuclear maturation rates. Further analysis of the effects of GO on the oocytes were explored via confocal microscopy (Nikon AX/AXR system). The results indicated that despite its small size, GO did not penetrate the zona pellucida and thus, was unable to enter the oocyte cytoplasm (Fig 3). These findings ***confirm the safety of GO when used in conjunction with female gametes*** *in vitro.*

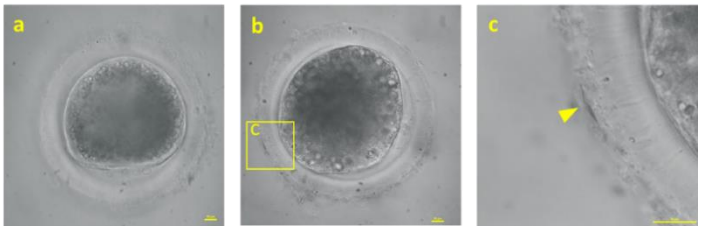

Figure 3: ***a.*** *Oocyte cultured without GO.* ***b.*** *Oocyte cultured in the presence of GO.* ***c.*** *Detail of the presence of GO sheets attached to the outside of the zona pellucida. Scale bars represent 10 μm.*

(3) Analysis of GO and rGO as ROS quenching cryoprotectant supplements.

Next, we intended to elucidate if GO and rGO were able to contribute to protect oocytes during cryopreservation by the Cryotop® method, a widely used commercial method (Kitazato). When the materials were applied to the device surface prior to freezing, the oocytes exhibited lower viability compared to the control group, particularly with rGO. This outcome is attributed to ***strong cell adhesion to surfaces covered with GO and rGO, resulting in significant mechanical damage*** *during thawing,*

leading to cell death. Conversely, when the materials were used during the recovery phase after thawing, they had less impact on causing cell damage. While GO showed a slight positive effect on preserving cell viability, *it did not differ significantly from the control group.*

(4) Analysis of the reduced ROS quenching activity of GO in an in vitro culture setting.

The lack of significant results supporting the role of GO as an effective cryoprotectant prompted us to investigate its potential interaction with other components in the culture media, possibly explaining its poor performance in ROS quenching. Our analysis focused on the ability of pristine GO to bond with both inorganic and organic elements present in the culture media used in our experiments. EDS-SEM analysis confirmed the strong affinity of GO for positively charged ions like Mg, K, or Na found in the inorganic salts of the culture media. Additionally, FT-IR analysis showed robust bonding between GO and essential and non-essential amino acids.

These results indicate that ***GO is safe to use in an in vitro culture setting.*** *Moreover, with this study we suggest that the surface of* ***GO must be further modified with other molecules to enhance its performance as a ROS quencher.*** *The development of new GO-based nanomaterials could enhance not only the capacity of this material to capture ROS effectively in a long-term in vitro culture setting, but also could allow it to be used for other purposes.*

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 Ferre-Pujol Pilar, Ortiz-Anaya Israel, Yang Zhou, Nishina Yuta
２．発表標題 50th International Embryo Technology Society meeting
３．学会等名 Graphene-oxide toxicity evaluation and its effects on porcine oocyte in vitro maturation（国際学会）
４．発表年 2024年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関