

## Effects of liraglutide on sperm characteristics and fertilization potential following experimentally induced diabetes in mice

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### Article Info

#### Article history:

Received: 31 October 2018  
Accepted: 01 June 2019  
Available online: 15 March 2021

#### Keywords:

Experimental diabetes  
Fertilizing potential  
Liraglutide  
Sperm parameters

### Abstract

The current study was conducted to analyze the dose-dependent effects of liraglutide against the diabetes-induced detrimental impact on sperm parameters and fertilization potential. For this purpose, 42 adult male mice were randomly divided into control (with no intervention) and experimental groups. Next, the experimental group was subdivided into diabetic, 1.20 mg kg<sup>-1</sup> liraglutide-received diabetic, 1.80 mg kg<sup>-1</sup> liraglutide-received diabetic, 1.20 mg kg<sup>-1</sup> liraglutide-received non-diabetic and 1.80 mg kg<sup>-1</sup> liraglutide-received non-diabetic groups. All chemicals were administrated subcutaneously. Following 42 days, the animals were euthanized, and sperm samples were collected. The sperm count, motility, viability, DNA integrity, and maturity were analyzed and compared between groups. Moreover, the sperm fertilization potential was investigated by *in vitro* fertilization (IVF). For this purpose, the preimplantation embryo development at 2-cell, 4-cell, morula, and blastocyst stages was investigated and compared. Observations revealed that diabetes significantly diminished sperm count, motility, viability, chromatin condensation, and DNA integrity percentages *versus* a control group. On the other hand, 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> of liraglutide did not improve sperm motility and viability, while ameliorated sperm count and chromatin condensation and DNA integrity in diabetic animals. The diabetic animals represented diminished preimplantation embryo development, which was not altered in liraglutide-received groups. In conclusion, at least in administrated doses, liraglutide could not improve the sperm viability and motility and, via this mechanism, could not induce an appropriate/beneficial effect on IVF outcome.

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### Introduction

During the last few decades, several clinical observations and meta-analyses in the USA and Europe have shown that different factors can affect male reproductive potential negatively.<sup>1</sup> Among these factors, diabetes mellitus (DM) and type II diabetes, as a prevalent metabolic disorder, is shown to affect different organs by systemic hyperglycemia induction detrimentally.<sup>2</sup> For instance, retinopathy, neuropathy, nephropathy<sup>3</sup> and accelerated macro-vascular, and micro-vascular<sup>4</sup> disorders are reported as the main complications for DM. Similar to other organs, DM can negatively affect the male reproductive system. Previous studies have reported that DM pathologically affects spermatogenesis via suppressing testosterone synthesis,<sup>5</sup> inducing oxidative stress<sup>6</sup> and

triggering the mitochondria-dependent apoptosis,<sup>7</sup> and it can negatively affect preimplantation embryo development, as well.<sup>8</sup> Minding the positive correlation between insulin resistance and obesity, DM can potentially affect the sperm DNA integrity, consequently resulting in temporal and complete infertility.<sup>5,9</sup>

Thus, based on mentioned findings, DM is an essential topic in man-related health care and medication.<sup>10</sup> In line with this issue, various therapeutic agents such as insulin and insulin mimic/tropic chemicals control the DM-induced hyperglycemia in DM patients. Among all these agents, since the years 2006-2007, the incretin-based medication is introduced in the USA and European medical society, which is known to effectively impact the total blood glucose level.<sup>11</sup> Incretins are hormones, which are secreted from enteroendocrine gut cells into the systemic circulation.

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Indeed, the incretins are categorized into glucose-dependent insulinotropic peptide and glucagon-like peptide-1 (GLP-1), exerting different interactions in and outside of the pancreas. Among various physiological roles of GLP-1, this hormone, by exhibiting insulinotropic effect, regulates the insulin amount within minutes after food consumption.<sup>12,13</sup> GLP-1, through inhibiting glucagon secretion, potentially enhances insulin biosynthesis, and via this mechanism, it can down-regulate the DM-induced hyperglycemia.<sup>14</sup> Based on the mentioned effects of GLP-1, a newly developed chemical named liraglutide is introduced and approved in the USA and Europe. With a chemical formula of  $C_{172}H_{265}N_{43}O_{51}$  and molar mass of 3751.202 g Mol<sup>-1</sup>. Liraglutide is an acylated GLP-1 receptor agonist containing sequences of amino acid 97.00% similar to endogenous human GLP-1.<sup>15</sup> Liraglutide inhibits glucagon secretion, increases glucose-stimulated insulin secretion, restricts appetite, and delays gastric emptying.<sup>15,16</sup>

Although several effects of liraglutide are reported, this agent's possible beneficial effect against DM-induced pathogenesis on sperm parameters is not clearly understood. Considering that patients with DM represent a diminished expression of GLP-1<sup>17</sup> and minding low semen quality in DM condition, here in the current study, the possible ameliorative effect of liraglutide, a GLP-1 agonist, was investigated in experimentally-induced DM condition by focusing on sperm parameters and fertilization potential.

## Materials and Methods

To follow-up, the current experimental study, 42 adult and healthy male mice (10 weeks of age) with an average weight of  $25.00 \pm 5.00$  g were procured from the authorized Laboratory Animal Center of Urmia Medical University, Urmia, Iran. For oocyte collection, female adult healthy virgin mice (8-10 weeks of age) were considered and obtained from the same center. Following one week adaptation (a standard condition of 12 hr light/12 hr dark, humidity and temperature), the experiments were initiated under the monitoring of the Ethical Committee of Urmia University for Animal Research and Care (No: AECVU-179-2018).

**Animals and grouping.** The animals were divided into control and experimental groups. The animals in the control group ( $n = 7$ ) received normal saline with no experimental intervention. Next, the animals in experimental group subdivided into DM ( $n = 7$ ), non-diabetic liraglutide-received  $1.20 \text{ mg kg}^{-1}$  (Novo Nordisk Pharmaceuticals, Taguig, Philippines) received ( $n = 7$ ), non-diabetic  $1.80 \text{ mg kg}^{-1}$  liraglutide-received ( $n = 7$ ), diabetic  $1.20 \text{ mg kg}^{-1}$  liraglutide-received ( $n = 7$ ) and  $1.80 \text{ mg kg}^{-1}$  liraglutide-received ( $n = 7$ ) groups.<sup>2</sup> The liraglutide was administrated subcutaneously for 42 consecutive days (every day).

**Diabetes induction.** Following 12 hr of food deprivation but free access to water, the experimental DM was induced in diabetic and liraglutide-received diabetic groups. For this purpose,  $200 \text{ mg kg}^{-1}$  of streptozotocin (Sigma-Aldrich, St. Louis, USA) was dissolved in 20.00 mmol freshly prepared citrate buffer (Sigma-Aldrich) and then injected intraperitoneally (IP).<sup>18-20</sup> After 18 hr, the blood glucose levels were detected by glucometer (Beurer GmbH, Ulm, Germany) through a tail puncture. The animals with blood glucose levels higher than  $200 \text{ mg dL}^{-1}$  were considered diabetic. The blood glucose was evaluated in 2 days interval for 42 days. Following 42 days, the animals were euthanized by ketamine ( $80 \text{ mg kg}^{-1}$ ; Alfasan, Woerden, The Netherlands) and xylazine ( $10.00 \text{ mg kg}^{-1}$ ; Phoenix Pharmaceuticals, St. Joseph, USA) cocktail injection<sup>21</sup> followed by cervical dislocation. Next, the epididymis was dissected out.

**Sperm analyses.** To evaluate the sperm quality parameters, the right cauda epididymis was placed in 500  $\mu\text{L}$  human tubular fluid culture medium or HTF; containing 4.00 mg bovine serum albumin (Sigma-Aldrich). Next, the tissue was cut into 2-3 pieces, and after that, to allow sperm to swim up, the sample was incubated at  $37.00 \text{ }^\circ\text{C}$  for 60 min in a  $\text{CO}_2$  incubator (LEEC, Nottingham, UK).<sup>22,23</sup>

**Epididymal sperm count.** The epididymal sperm were counted based on the hemocytometer method.<sup>24</sup> For this purpose, the sperm suspension was diluted in distilled water (1:20), and an aliquot of 10.00  $\mu\text{L}$  was transferred to each counting chamber of the hemocytometer. Following 5 min, the sperm count was performed using a light microscope at 400 $\times$ . The sperm count was presented as a number of sperm per mL which was obtained using the following formula:

$$\text{Sperm cell number} = n \times 50,000 \times d$$

where,  $n$  = the counted sperm number and  $d$  = reverse of sperm suspension dilution.

**Sperm viability assessment.** A 20.00  $\mu\text{L}$  aliquot of the sperm suspension was mixed with an equal volume of 0.05% eosin-Y ( $37.00 \text{ }^\circ\text{C}$ ). After 20-30 sec, 20.00  $\mu\text{L}$  of nigrosin was added, and following 2 min incubation at room temperature, the slides were analyzed under a bright-field microscope (400 $\times$ ). The dead sperms were revealed with pink-stained cytoplasm, and the live cells remained unstained. Finally, 200 cells were counted for each sample, and viability percentages were calculated.<sup>25</sup>

**Sperm motility evaluation.** To determine the sperm motility percentage, a drop of the sperm-containing suspension was placed on a glass slide ( $37.00 \text{ }^\circ\text{C}$ ), and coverslipped. At least five microscopic fields (400 $\times$ ) were evaluated for each sample, and the average percentages of motile sperms were recorded for all groups.<sup>26</sup>

**Determination of sperms with damaged DNA.** The acridine-orange staining technique was used to evaluate

sperm DNA integrity. Based on DNA integrity, the acridine-orange staining technique classifies the sperms into sperms with double- and single-strand DNA representing green and red fluorescent reactions, respectively. For this purpose, a drop of sperm-containing culture medium was spread on the glass slides and allowed to be air-dried. All smears were fixed in methanol/acetic acid (3:1). The slides were then stained with 19.00% acridine-orange (Sigma-Aldrich) solution in phosphate citrate for 10 min. The sperms were evaluated with a fluorescence microscope (GS7; Nikon, Tokyo, Japan) with a 40 $\times$ .<sup>27</sup>

**Sperm nuclear maturation (chromatin quality) examination.** For the determination of sperm chromatin maturation, the aniline-blue staining technique was considered. The technique is based on a protamine-histone replacement process, in which the sperms with intact protamination represent darkly stained nuclei (sperms with mature chromatin), and those with unstained nuclei are considered as sperms with impaired protamine-histone replacement (sperms with immature chromatin). For this purpose, a drop of the sperm-containing medium was smeared on glass slides and stained with an acidified 5.00% aniline-blue solution (pH: 3.50). Next, the slides (5-6 min at room temperature) were washed and allowed to be dried. An average of 200 cells was counted on each slide, and the percentage of sperms with immature chromatin was evaluated and compared between groups.<sup>28</sup>

**Oocyte retrieval.** As mentioned above, healthy adult female mice were considered for oocyte retrieval. For this purpose, the pregnant mare's serum gonadotropin (Focus Bioscience, Brisbane, Australia) was injected (10.00 IU per animal, IP), followed by an injection of human chorionic gonadotropin (hCG; 10.00 IU per animal, IP) after 48 hr. Following 10 to 12 hr from hCG (Bioscience GmbH, Dümmer, Germany) injection, the animals were anesthetized with ketamine (80.00 mg kg<sup>-1</sup>) and xylazine (10.00 mg kg<sup>-1</sup>) cocktail and euthanized by cervical disarticulation. The ampullae of oviducts were removed, transferred to a Petri dish containing 500  $\mu$ L HTF (Irvine Scientific Inc., Santa Ana, USA) supplemented with 4.00 mg mL<sup>-1</sup> bovine serum albumin (Sigma-Aldrich) and incubated at 37.00 °C with 5.00% CO<sub>2</sub>. After that, the oocytes were retrieved from oviducts through direct dissection under a stereomicroscope (T12; Olympus Co., Tokyo, Japan).<sup>29</sup>

**In vitro fertilization.** The isolated sperms belonged to all groups pre-incubated for 1 hr in HTF containing gentamicin sulfate 10.00  $\mu$ g mL<sup>-1</sup> and 4.00 mg mL<sup>-1</sup> bovine serum albumin at 37.00 °C in an atmosphere of 5.00% CO<sub>2</sub> to ensure capacitation. A volume of 0.10 mL of sperm suspension (containing 10<sup>6</sup> mL<sup>-1</sup> sperms) was introduced into a 0.90 mL fertilization drop of oocytes-containing HTF. For each animal, a total of 20 oocytes were divided into 10 drops (four animals, 80 oocytes in 40 drops). All fertilization steps and embryo culture were carried out

under detoxified mineral oil. The zygote formation was verified after 4-6 hr from sperm exposure in each group, and the results were represented in percentages and compared between groups.

**Statistical analyses.** For all numerical results, the mean and standard error of the measured parameters were calculated. The results were analyzed using SPSS Software Package for Windows (version 16.0; SPSS, Chicago, USA). The comparisons between groups were made by one-way analysis of variance followed by Bonferroni post hoc test. A value of  $p < 0.05$  was considered significant.

## Results

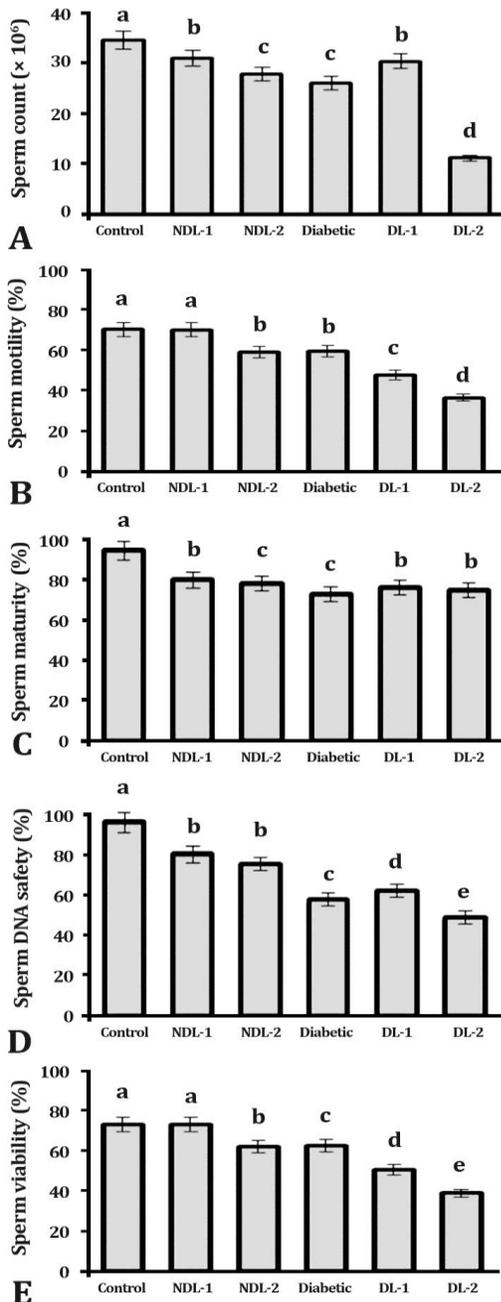
**Sperm count.** The animals in the experimental group (diabetic, non-diabetic and diabetic liraglutide-received) represented a significant ( $p < 0.05$ ) reduction in sperm count *versus* control animals. No significant differences were revealed between non-diabetic liraglutide-received animals ( $p > 0.05$ ). The lowest sperm count was revealed in the 1.80 mg kg<sup>-1</sup> liraglutide-received diabetic group. Accordingly, the diabetic animals received 1.20 mg kg<sup>-1</sup>, and 1.80 mg kg<sup>-1</sup> liraglutide exhibited a remarkable reduction in sperm count than the diabetic and control animals (Fig. 1A).

**Sperm motility.** No significant differences were revealed between control and non-diabetic 1.20 mg kg<sup>-1</sup> liraglutide-received animals ( $p > 0.05$ ). However, the other experimental groups exhibited decreased sperm motility *versus* control and non-diabetic 1.20 mg kg<sup>-1</sup> liraglutide-received groups. The mice in diabetic 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> liraglutide-received groups exhibited diminished sperm motility ( $p < 0.05$ ) *versus* diabetic animals (Fig. 1B).

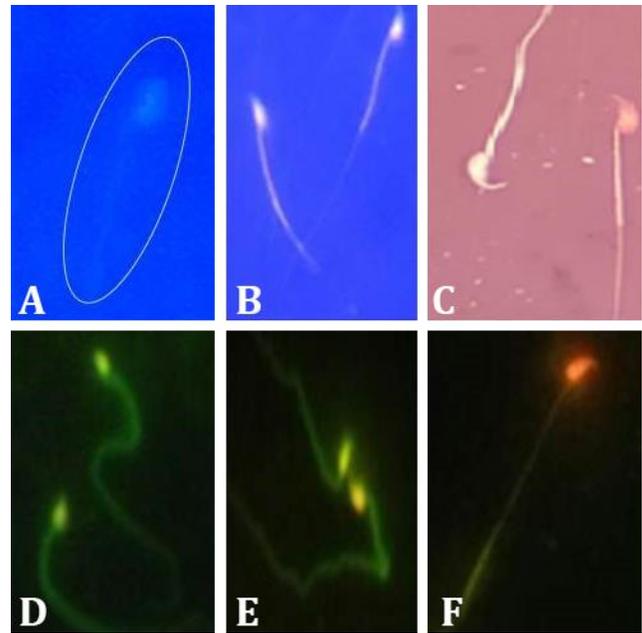
**Sperm chromatin condensation and DNA integrity.** Despite sperm count and motility percentage, the diabetic animals in both 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> liraglutide-received groups represented a significant ( $p < 0.05$ ) improvement in sperm chromatin condensation (Fig. 1C). Hence, the animals in these groups revealed remarkably higher percentages of sperms with condensed chromatin *versus* the diabetes group. No significant changes were demonstrated between non-diabetic and diabetic liraglutide-received groups ( $p > 0.05$ ). About DNA integrity, the animals in experimental groups represented a significant ( $p < 0.05$ ) reduction in the percentage of sperms with intact DNA integrity *versus* control animals. The lowest percentage of DNA integrity was revealed in diabetic mice, which was significantly ( $p < 0.05$ ) improved in 1.20 mg kg<sup>-1</sup> liraglutide-received group (Fig. 1D).

**Sperm viability.** No significant difference was revealed between control and non-diabetic 1.20 mg kg<sup>-1</sup> liraglutide-received groups ( $p > 0.05$ ). However, the percentages of sperm viability were decreased in other experimental groups compared to control animals.

Comparing the results between diabetic and 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> liraglutide-received diabetic mice showed a remarkable ( $p < 0.05$ ) reduction in sperm viability of liraglutide-received animals *versus* the diabetes group (Fig. 1E). Figure 2 also represents the photo-micrographs of different used staining techniques for sperm analyses.



**Fig. 1.** Effects of different treatments on sperm parameters including **A)** Sperm count; **B)** Sperm motility; **C)** Sperm maturity; **D)** Sperm DNA safety; **E)** Sperm viability. NDL-1: Non-diabetic liraglutide 1.20 mg kg<sup>-1</sup>; NDL-2: Non-diabetic liraglutide 1.80 mg kg<sup>-1</sup>; DL-1: Diabetic liraglutide 1.20 mg kg<sup>-1</sup>; DL-2: Diabetic liraglutide 1.80 mg kg<sup>-1</sup>. Different superscripts designating significant differences ( $p < 0.05$ ).



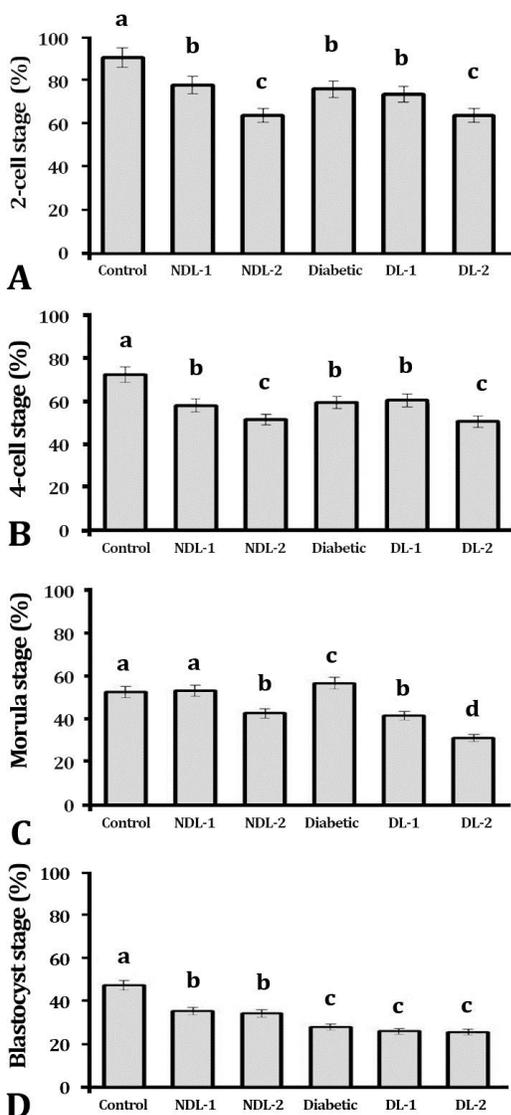
**Fig. 2.** **A)** Immature sperm with low protamine and high histone is dark blue (sperm outlined; aniline-blue; 400×); **B)** Mature sperms with high protamine are bright or light blue (aniline-blue; 400×); **C)** Live sperm is white and dead one is red (Eosin-Nigrosin; 400×); **D, E, and F)** Sperms with DNA damage are yellow to orange, and those with intact DNA are green (Acridine orange; 400×).

#### Two- and four-cell embryo development.

Observations revealed a significant ( $p < 0.05$ ) reduction in percentages of 2- and 4-cell embryos in the experimental groups *versus* the control one (Figs. 3A and 3B). No significant differences were revealed in percentages of 2- and 4-cell embryos between diabetic, non-diabetic 1.20 mg kg<sup>-1</sup> liraglutide and diabetic 1.20 mg kg<sup>-1</sup> liraglutide-received animals ( $p > 0.05$ ).

#### Morula and blastocyst development.

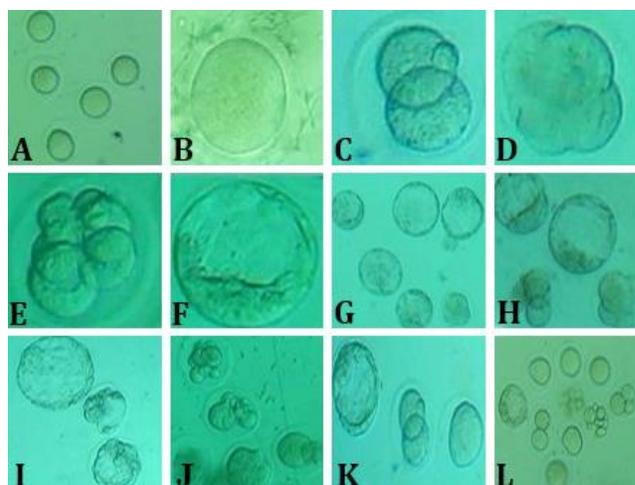
No significant change was demonstrated for a percentage of morula between control and non-diabetic 1.20 mg kg<sup>-1</sup> liraglutide-received groups ( $p > 0.05$ ). However, the liraglutide significantly decreased the percentage of morula in the non-diabetic 1.80 mg kg<sup>-1</sup> liraglutide-received group ( $p < 0.05$ ). The other experimental groups exhibited a significant reduction in percentages of morula *versus* control and non-diabetic 1.20 mg kg<sup>-1</sup> liraglutide-received animals ( $p < 0.05$ ). The lowest percentage of morula was revealed in diabetic 1.80 mg kg<sup>-1</sup> liraglutide-received mice (Fig. 3C). Similar to other results, the animals in all experimental groups represented a remarkable ( $p < 0.05$ ) reduction in the percentage of blastocyst *versus* the control group (Fig. 3D). No significant differences were revealed between diabetes and diabetic 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> liraglutide-received groups ( $p > 0.05$ ). Figure 4 represents different stages of preimplantation embryos in different groups.



**Fig. 3.** Effects of different treatments on embryo development including **A)** 2-cell stage; **B)** 4-cell stage of embryo; **C)** Morula stage of embryo; **D)** Blastocyst stage of embryo. NDL-1: Non-diabetic liraglutide 1.20 mg kg<sup>-1</sup>; NDL-2: Non-diabetic liraglutide 1.80 mg kg<sup>-1</sup>; DL-1: Diabetic liraglutide 1.20 mg kg<sup>-1</sup>; DL-2: Diabetic liraglutide 1.80 mg kg<sup>-1</sup>. Different superscripts designating significant differences ( $p < 0.05$ ).

## Discussion

According to the WHO report, the DM is a chronic metabolic disorder, and its prevalence will rise by 2030.<sup>30</sup> Insulin secretion failure or diminished perceptivity of target tissues to the metabolic effects of insulin is known as the main reasons for DM.<sup>31-33</sup> As a result, DM can impact fertility potential in both genders.<sup>34-37</sup> Different synthetic and herbal agents, including insulin (at the top), DPP-4 inhibitors, meglitinides, sulfonylureas, metformin, and GLP-1 receptor agonists, administered to ameliorate or inhibit the DM-induced detrimental impact on different organs.



**Fig. 4.** **A)** Oocytes; **B)** Fertilization; **C)** 2-cell embryos; **D)** 4-cell embryos; **E)** Morula; **F)** Blastocysts. **G)** Control; **H)** Non-diabetic liraglutide 1.20 mg kg<sup>-1</sup>; **I)** Non-diabetic liraglutide 1.80 mg kg<sup>-1</sup>; **J)** Diabetic; **K)** Diabetic liraglutide 1.20 mg kg<sup>-1</sup>; **L)** Diabetic liraglutide 1.80 mg kg<sup>-1</sup>.

Among several therapeutic agents, here in the current study, we focused on liraglutide (a GLP-1 agonist agent) against DM-induced detrimental effect on sperm parameters, fertilization potential, and preimplantation embryo development. It has been well-established that experimentally-induced DM in the animal model significantly impacts male reproductive potential by Sertoli cell vacuolization,<sup>38</sup> and diminishing Leydig, Sertoli, spermatogonium cells in testicular tissue. Via this mechanism(s), it negatively affects the spermatogenesis and spermiogenesis processes leading to a severe reduction in sperm concentration.<sup>38-40</sup> In corroboration with these reports, we revealed a significant reduction in sperm count of diabetic animals. The interesting point was that we failed to see a remarkable enhancement in 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> liraglutide-received diabetic animals. It means that liraglutide, despite its insulin-mimic characteristics, is not able to recover the sperm count. Minding the diabetes-induced mitochondria-dependent apoptosis at the germ cell level,<sup>7,37</sup> seems that liraglutide has not been able to recover the spermatogenesis or spermiogenesis processes. Diabetes, at least in animal model studies, significantly suppresses testicular antioxidant status. Indeed, diabetes-induced hyperglycemia increases NADPH consumption. Considering the co-factor role of NADPH in glutathione regeneration, hyperglycemia results in redox stress.<sup>41-44</sup> On the other hand, free radicals and reactive oxygen species (ROS) can target the protein, lipid, and nucleotide content of cells, consequently inducing severe DNA damage and lipid and protein peroxidation.<sup>44,45</sup> Here, in the current study, we demonstrated the same pattern. Accordingly, the animals in the diabetes group exhibited a significant reduction in the percentage of sperms with intact DNA content.

However, the 1.20 mg kg<sup>-1</sup> liraglutide could fairly up-regulate the percentage of sperms with intact DNA content. Minding the negative effect of 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> of liraglutide on non-diabetic animals as well as 1.80 mg kg<sup>-1</sup> of liraglutide on diabetic animals, we can come close to this fact that it can ameliorate the hyperglycemia-induced DNA fragmentation, only when it is administrated in diabetic condition with a dose level of 1.20 mg kg<sup>-1</sup>. Otherwise, administration of liraglutide or higher doses of this chemical solely can even enhance the DNA fragmentation. All these impacts may be related to liraglutide inability to recover the testicular antioxidant status and/or liraglutide administration route, leading to partial suppressive redox system. Considering the impact of liraglutide on circular and testicular glucose levels, it is logical to conclude that in physiological condition, liraglutide could negatively affect the normal metabolic system of germ cells. Therefore, an impaired metabolism, in turn, results in high amounts of free radical production. It alters the balance between antioxidant/oxidant system in testicles, which in turn may cause DNA fragmentation and immobility of sperm cells. However, to better understand the issue, more experiments are needed to focus on testicular antioxidant status after liraglutide consumption. Despite the hypotheses mentioned above, we assessed the DNA compaction by aniline-blue staining to determine the possible role of epigenetic in liraglutide (1.80 mg kg<sup>-1</sup>) failure to maintain sperm DNA integrity. To understand the subject, it should be noted that the histone-protamine replacement protects the sperm DNA content against ROS-induced damages.<sup>46,47</sup> It means that in the case of impaired protamine replacement, the chromatin content of sperms will be sensitive to free radicals, and ROS will easily damage it. Our findings revealed that diabetes significantly suppressed the replacement process marked with a decreased percentage of sperms with chromatin condensation. On the other hand, liraglutide (especially at a dose level of 1.20 mg kg<sup>-1</sup>) could fairly ameliorate the chromatin condensation marked by aniline-blue staining. This finding shows that liraglutide could improve the chromatin condensation, and via this mechanism could recover the sperm DNA integrity. Although sperm chromatin condensation and DNA integrity are known as two important parameters, sperm viability is another essential parameter in evaluating fertilization potential. Thus, the percentages of sperm viability were evaluated and compared between groups. Observations showed that 1.20 mg kg<sup>-1</sup> of liraglutide did not affect the sperm viability when it was administrated solely. However, the animals in the other liraglutide-received groups (1.80 mg kg<sup>-1</sup> non-diabetic, 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> diabetic groups) exhibited a significant reduction in percentages of viable sperms. Although the mechanism(s) by which the ROS adversely affected the sperm motility was/were not assessed in the present

study, impaired axonemal protein phosphorylation and free radicals diffusion across sperm membrane are generally known for ROS-induced adverse impacts on sperm motility.<sup>48,49</sup> Here in the current study, we revealed that the liraglutide could not improve sperm motility and the diabetic animals in 1.20 mg kg<sup>-1</sup> and 1.80 mg kg<sup>-1</sup> liraglutide-received groups represented diminished percentages of motile sperms *versus* the diabetes group. Thus, we can suggest that, despite the protective effect of liraglutide on sperm DNA content, it was not able to maintain sperm viability and motility.

To determine the exact effect of liraglutide on fertilization potential, IVF was considered in the current study. We found that diabetes significantly decreased the 2- and 4-cell embryo development, while it was not statistically significant compared to 1.20 mg kg<sup>-1</sup> liraglutide diabetic and non-diabetic animals. Both diabetic and non-diabetic 1.80 mg kg<sup>-1</sup> liraglutide-received animals represented lower percentages of 2- and 4-cell embryos *versus* the diabetes group. The results for blastocysts formation, the most important outcome of IVF, showed that liraglutide at both dose levels could not up-regulate the percentages of blastocysts. Salamun and co-workers have shown that liraglutide and metformin can remarkably improve IVF outcomes in obese women with polycystic ovary syndrome.<sup>49</sup> In contrast to their findings, here in the current trial, we found that liraglutide exerts no significant effect on IVF outcomes. This situation may be related to its inability in up-regulating the sperm viability and/or motility. Because the immotile sperms with damaged DNA are not able to initiate intact and well-progressing preimplantation embryo development.<sup>45</sup>

Our data showed that administration of liraglutide, at least in animal models, adversely affects the sperm parameters in a dose-dependent manner. Moreover, we found that administering 1.20 mg kg<sup>-1</sup> liraglutide fairly protects the sperm chromatin and DNA integrity via improving the chromatin condensation process in diabetic animals. However, at a higher dose level (1.80 mg kg<sup>-1</sup>), it negatively affects sperm DNA content in sole and diabetic conditions. It is shown that liraglutide at both dose levels does not effectively protect sperm viability and motility potentials in diabetic conditions. Finally, we demonstrated that liraglutide in sole form and in diabetic condition inhibits the preimplantation embryo development.

### Acknowledgments

The authors express their appreciation to the Faculty of Veterinary Medicine, Urmia University, and Faculty of Medicine, Urmia University of Medical Sciences for their kind scientific aids. We are also grateful to staff members of Histology and Embryology and Physiology Laboratories, particularly Dr. Ali Karimi and Dr. Naseh Abdullahzadeh for their kind help.

## Conflict of interest

The authors declare that they have no conflict of interest regarding the publication of this article.

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