Journal of Assisted Reproduction and Genetics (2021) 38:227–233
https://doi.org/10.1007/s10815-020-01999-w

GAMETE BIOLOGY

Three hour abstinence as a treatment for high sperm DNA fragmentation: a prospective cohort study

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Received: 18 May 2020 / Accepted: 28 October 2020 / Published online: 12 November 2020
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Abstract

Purpose This study sought to compare sperm DNA fragmentation (SDF) in semen specimens after 3 days and then after 3 h of abstinence in men presenting for initial infertility evaluation.

Methods A prospective cohort study of 112 men undergoing their first semen analysis as part of an infertility work-up was conducted. All participants presented with 3 days of abstinence for a semen analysis and DNA-fragmentation test. Both tests were repeated on a second sample collected 3 h after the first ejaculation. DNA-fragmentation was evaluated with the halo test by one of two technicians blinded to duration of abstinence. Variables analyzed include ejaculate volume, sperm concentration and motility, smoking status, cannabis use, initial specimen DNA fragmentation, and use of sperm-directed anti-oxidant formulations.

Results Among all subjects, DNA fragmentation improved in the 3-h abstinence specimen (34.6 ± 19.4% vs. 23.7 ± 16.0%, p = 0.0001). Among subjects with high DNA fragmentation (> 35%) on the initial specimen, 55% improved into the normal range. Semen volume and sperm concentration decreased (3.1 ± 3.3 ml vs. 1.9 ± 0.8 ml, $p < 0.01$ and 41 ± 39 vs. 32 ± 31 (millions/ml), $p = 0.01$), while progressive motility tended to increase. Fifty-eight subjects demonstrated ≥ 30% improvement in SDF in the second specimen as compared to the first. Factors found to correlate with > 30% improvement in DNA fragmentation in the 3-h abstinence specimen compared to 3 days were younger age and use of anti-oxidants.

Conclusion High SDF can often be managed with a second ejaculation 3 h after the first in infertile couples, including in males with abnormal semen analyses per the 2010 WHO guide. Apart from SDF levels, changes in sperm quality were not clinically significant in the second specimen and did not increase rates of ICSI. However, a second ejaculation after 3 h probably may reduce the necessity of costly and/or invasive ART strategies.

Keywords Semen analysis · DNA fragmentation · Abstinence · IVF · IUI

Introduction

Sexual abstinence is one of the several factors known to influence semen parameters and is one of the clinical criteria included in the World Health Organization (WHO) semen evaluation guidelines to provide maximum sperm quality [1]. The WHO recommends a period of abstinence ranging from 2 to 7 days prior to semen collection for standard evaluation, whereas the European Society for Human Reproduction and Endocrinology (ESHRE) and the Nordic Andrology Association (NAA) suggest tighter abstinence of 3–4 days [2]. The standardization of sexual abstinence is traditionally recommended as a strategy to permit inter-laboratory homogenization of results [3]. However, there is no clear scientific evidence that a prolonged period of abstinence is useful to achieve greater success with the use of artificial reproductive

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technologies. In fact, there are some studies which have shown that prolonged abstinence can have negative effects on semen quality [4, 5]. It is well accepted that semen volume and sperm concentration will increase with prolonged abstinence, but such abstinence can have a negative impact on motility and viability [3].

Spermatozoa must pass through the epididymis in order to undergo a series of physiological and biochemical changes that allow them to acquire fertilizing potential [6]. During this transit and storage period, spermatozoa are known to be exposed to a higher concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) than elsewhere in the reproductive tract [7–9]. It has been well described in studies of ROS production and sperm physiology that these highly reactive chemicals play a major role in important sperm processes such as maturation, motility, and fertilization capacity [10]. Male germ cells are susceptible to the accumulation of DNA lesions because to allow for quick and effective induction of DNA double strand breaks for meiotic crossovers, DNA repair capacity declines during the latter part of spermatogenesis [2, 11, 12]. As a consequence, sperm can quickly accumulate DNA damage and fragmentation during storage in the epididymis and the subsequent exposure to ROS [2, 13, 14]. When compared to ejaculated sperm, testicular sperm shows a lower level of sperm DNA fragmentation (SDF), suggesting that SDF is increased between transit from the seminiferous tubules and eventual ejaculation [11, 15]. In addition to exposure to ROS in the epididymis, other causes of SDF include defective apoptosis, the toxic effects of drugs, cigarette smoking, pollution, high testicular temperature (fever or varicocele), and advancing age [2]. While low SDF does not guarantee normal male fertility, the chance of a spontaneous conception declines with SDF levels > 20% and lower levels still for values > 30–40%, depending on the assay used [9, 16, 17]. Furthermore, levels of SDF were comparable between idiopathic sub-fertile men with normal sperm parameters and sub-fertile men with abnormal sperm parameters but were significantly higher in both of these groups compared to fertile controls [18, 19].

Since oxidative stress in the epididymis is considered the main cause of increased SDF, then with more prolonged storage time, one would expect higher SDF. As a result, some studies have assessed the effect of reduced abstinence times on the levels of SDF in semen samples. Mayorga-Torres et al. demonstrated that when compared to a 3–4 day abstinence period, there was a reduction in the levels of intracellular ROS in semen samples collected after 24 h of abstinence [20]. In the same study, the researchers also examined the effects of daily ejaculation frequency on traditional semen parameters and noted that with daily ejaculation, while seminal volume and total sperm count were reduced, there was no major change in motility, viability, or morphology. They specifically noted that daily ejaculations did not drop sperm concentration below WHO reference values [20]. In 2014, Marshburn et al. confirmed the finding that a 1-day abstinence period improved sperm quality by protecting from ROS damage [21]. However, this study demonstrated that a lower number of sperm was produced when compared to a longer abstinence period [21]. Finally, a study looking specifically at SDF fragmentation with shorter abstinence periods suggested that a short 3-h abstinence period provided a 31% effective reduction in SDF when compared to 24 h of abstinence in a cohort of twelve normozoospermic men [22] .

Low SDF is considered an important factor in achieving a healthy pregnancy. Therefore, different strategies have been used to reduce the impact of a high SDF in the ejaculate for ART. Currently, these strategies include in vitro direct selection of sperm containing undamaged DNA (ANEXIN separation), ingestion of certain drugs that may decrease rate of SDF, and testicular sperm retrieval (TESA) [22, 23]. These strategies are either highly invasive, require advanced levels of ART such as intracytoplasmic sperm injection (ICSI), or both. It should also be acknowledged that they add cost to the procedures and with the exception of drug ingestion can only be performed at IVF and not with intra-uterine insemination. Given the findings of recent studies on the positive impact of shorter abstinence times on SDF, more robust studies are needed to determine the optimal time for abstinence in patients undergoing ART and how these improvements can affect ART options for patients [21, 22]. Currently, a 3-h abstinence period to reduce SDF has only been evaluated in a single study, in a small group of twelve men. The objective of this study, therefore, was to compare sperm DNA fragmentation (SDF) in semen specimens after 3 days and 3 h of abstinence in a larger cohort of men presenting for their first semen evaluation as part of an infertility work-up.

Materials and methods

This is a prospective cohort study of 112 patients who were undergoing fertility treatment at a private academically based infertility center from January 01, 2017, until June 30, 2019. Inclusion criteria were all males who were members of an infertile couple and presenting for their first semen analysis at this institution. Exclusion criteria were known or detected azoospermia or severe oligospermia preventing the use of the DNA fragmentation test or inability to produce a second semen analysis 3 h after the first ejaculation.

Parameters evaluated included DNA fragmentation, volume, motility, and concentration and total sperm count. Strict morphology was only evaluated on the first specimen after 3 days of abstinence. Analysis was performed on neat sperm; it had not undergone gradient separation.

Each subject was asked to abstain from ejaculation for 3 days prior to providing the first sample. A second sample was

then obtained from all participants 3 h after their first ejaculation. SDF was evaluated using the HaloSperm Assay (Microptic Inc, Barcelona, Spain) (sperm chromatin dispersion test) by one of two experienced andrology technicians. Both andrology technicians were blinded to whether the specimen was produced after 3 days or 3 h of abstinence.

All reagents for the following procedure were obtained from Microptic Inc (Barcelona, Spain) DNA fragmentation using the halo method: The method is based on the sperm chromatin dispersion (SCD) test as described by Fernández et al. [24]. Intact unfixed spermatozoa (fresh, frozen/unthawed, diluted samples) were immersed in an inert agarose microgel on a pretreated slide. An initial acid treatment was used to denature the DNA in those sperm cells with fragmented DNA. Following this, a lysing stock solution was applied which removed the majority of the nuclear proteins. In the absence of massive DNA breakage, this treatment produces nucleoids with large halos of spreading DNA loops, emerging from a central core. However, the nucleoids from spermatozoa with fragmented DNA either did not show a dispersion halo or the halo produced is minimal.

All semen analysis parameters were analyzed by SCA CASA System (Microptics Inc, Barcelona, Spain). The SCA CASA System for semen analysis allows the accurate, repetitive, and automatic assessment of the following sperm parameters: motility, concentration, and morphology. Results were confirmed with visual inspection and manual calculation by the laboratory technicians.

Some of the males took a sperm improvement anti-oxidant containing multi-vitamin which consisted of L-carnitine; vitamins A, C, E, and B12; selenium; and zinc (Yadtech Inc, Cote-St-Luc, Canada). This supplement was chosen because at the start of the study it was the only male fertility-related supplement sold in Canada. Although, it was prescribed to more subjects, we were able to capture those who took it. None of the physicians in neither our center nor any of the authors have any affiliations or interests in Yadtech, or this vitamin.

On the day of the semen analysis, subjects completed a questionnaire related to age, smoking status, cannabis use, and previous pregnancies. One hundred percent completed this questionnaire with nursing assistance.

Statistical evaluation was performed with intra-subject t tests. Data is presented as mean ± SD. Stepwise multivariate logistic regression was used to model predictors of ≥ 30% improvement in DNA fragmentation in the second specimen. This 30% cutoff was established arbitrarily. However, such an improvement was felt to be clinically important and as such was selected as a cutoff point for our model. Variables included in the model were age, anti-oxidant vitamin supplementation status, 1st and 2nd ejaculate volume, sperm concentration and motility, smoking status, cannabis use, and initial specimen DNA fragmentation. Power analysis suggested that ≥ 73 subjects were required for an 80% power and an alpha of 5% with a 2 unit mean difference with SD of 6 units.

All participants provided informed consent. IRB approval for this study was obtained. Subjects were not charged for the second semen and DNA fragmentation analysis. This study was funded by a grant from the Montreal Reproductive and Regenerative Medicine Foundation.

Results

One hundred and fifty-eight males had semen analysis ordered during the study period; 120 males came to produce the specimen; 112 males produced two specimens 3 h apart; 7 males did not return for the production of the second specimen. All males who attempted a second ejaculate 3 h after the first were successful in producing a sample for testing. Viagra was given if requested (six subjects). One male collapsed while in the production room and was found to have a fever of 101.4; he was transferred to the hospital where malaria was diagnosed, having recently returned from Africa. His specimen was also excluded from analysis.

There were a total of 112 patients with an average age of 41.1 ± 6.3 years (range 29–65). Among all subjects, DNA fragmentation improved from the 3-day to the 3-h abstinence sample (34.6 ± 19.4% vs. 23.7 ± 16.0%, $p \leq 0.0001$). Among subjects with high DNA fragmentation (≥ 35%) on the initial specimen (n = 49), 55% (27/49) improved into the normal range (sperm quality of 26 to 34% DNA fragmentation is referred to as borderline per the assay guide). The greatest improvement in a single individual was a decrease from 97 to 28% DNA-fragmentation. Six percent (7/112) of subjects had worse DNA fragmentation in the second specimen. However, all subjects where the DNA fragmentation got worse stayed within the normal range of the DNA fragmentation assay if they started out as normal in the 3-day abstinence specimen. Of those who got worse, only 2 fell above the normal range (1.8%), having also been higher than 35% DNA fragmentation in the initial specimen with 3 days abstinence. In none of the cases (N = 112) did the quality of the second semen specimen convert the subject to ICSI from a planned IVF without ICSI (Table 1), based on sperm analysis total count, concentration, and motility.

Table 2 outlines the results of the multivariate logistical regression analysis of factors that predict at least a 30% improvement in SDF in specimens produced after 3 h of abstinence. Only younger age (40.3 ± 6.0 vs. 42.1 ± 8.1 years, p = *0.03*, *95% CI 0.84–0.99*) and the use of sperm-directed anti-oxidants (34% vs. 17%, p = *0.02*, *95% CI 1.25–19.8*) were associated with a > 30% improvement in SDF. There was a trend to greater likelihood of 30% improvement in SDF with higher initial SDF percentage, although this was not significant (35.7 ± 18.4 vs. 33.2 ± 21.3, p = *0.06*, *95% CI 1.0–1.06*).

Table 1 Changes in semen parameters in specimens produced after 3 h of abstinence compared to 3 days of abstinence

	3 days abstinence $N = 112$	3 h abstinence $N = 112$	p value
Sperm DNA fragmentation (%) by halo	34.6 ± 19.4	23.7 ± 16.0	$p < 0.0001$
Volume (ml)	3.1 ± 3.3	1.9 ± 0.8	$p = 0.0001$
Concentration (millions/ml)	41 ± 39	32 ± 31	$p = 0.001$
Progressive motility (%)	57 ± 21	60 ± 21	$p = 0.06$
Strict morphology normal forms (%)	6.7 ± 3.2 range (0–16)	N/A	
Subjects with initial DNA fragmentation > 35% ($N = 49$)			
Sperm DNA fragmentation (%) by halo	52 ± 16	36 ± 17	$p < 0.0001$

N/A not available

Forty-four subjects had abnormal sperm parameters according to the 2010 WHO guidelines in their initial semen analysis. This included concentration less than 15 million per milliliter or progressive motility of less than 32% or total sperm count less than 39 million/specimen or strict morphology < 4% normal forms. In this group, the average male age was 42.6 ± 7.2 years. Among these subjects in the initial vs. the 3 h ejaculated specimen, DNA fragmentation improved from 40.0 ± 20.5 to 26.9 ± 15.9% ($p \leq 0.0001$), volume decreased from 3.4 ± 4.8 to 1.8 ± 0.8 ml ($p = 0.034$), concentration stayed stable at 18.7 ± 18.1 vs. 17.2 ± 17.3 million/ml ($p = 0.35$), and progressive motility increased from 45.8 ± 22.2 to 52.3 ± 21.8% ($p = 0.045$). The mean strict morphology normal forms were 1.9 ± 1.2% in the first specimen. It was not tested in the second specimen.

Sixty-eight subjects had normal sperm parameters according to the 2010 WHO guidelines in their initial semen analysis. This included concentration ≥ 15 million per milliliter or progressive motility ≥ 32% or total sperm count ≥ 39 million/specimen or strict morphology ≥ 4% normal forms. In this group, the average male age was 41.1 ± 6.9 years. Among these subjects in the initial vs. the 3-h ejaculated specimen, DNA fragmentation improved from 34.4 ± 18.7 to 24.0 ± 16.8% ($p \leq 0.0001$), volume decreased from 2.7 ± 1.2 to 1.9 ± 0.8 ml ($p \leq 0.0001$), concentration decreased at 49.7 ± 38.8 vs. 39.0 ± 31.8 million/ml ($p = 0.010$), and progressive motility increased from 60.7 ± 15.0 to 66.0 ± 15.1% ($p = 0.016$). The mean strict morphology normal forms were 7.9 ± 2.5% in the first specimen. It was not tested in the second specimen.

Discussion

The aim of this study was to compare sperm DNA fragmentation (SDF) in semen specimens after 3 days and 3 h of abstinence in a general population of male partners in infertile couple, including men with known male factor infertility. Previous studies which have demonstrated an improvement in SDF with shorter abstinence periods have only ever been done in a normozoospermic population [21, 22]. Furthermore,

Table 2 Factors which predict at least a 30% improvement in sperm DNA fragmentation in the specimens produced after 3 h of abstinence compared to 3 days of abstinence

	< 30% improvement in DNA fragmentation $N = 54$	≥ 30% improvement in DNA fragmentation $N = 58$	95% CI	p value
Male age (years)	42.1 ± 8.1	40.3 ± 6.0	0.84–0.99	*0.03*
Initial sperm DNA fragmentation (%)	33.2 ± 21.3	35.7 ± 18.4	1.0–1.06	*0.06*
Initial volume (ml)	2.6 ± 1.3	2.8 ± 1.2	0.84–2.65	NS
Second volume (ml)	1.9 ± 0.8	1.9 ± 0.8	0.23–1.39	NS
Initial concentration (millions/ml)	44.8 ± 37.4	39.7 ± 42.8	0.98–1.005	NS
Second concentration (millions/ml)	37.2 ± 28.8	37.1 ± 34.0	0.99–1.03	NS
Initial progressive motility (%)	56.1 ± 20.7	58.4 ± 21.0	0.97–1.03	NS
Second progressive motility (%)	58.9 ± 23.1	62.7 ± 19.1	0.98–1.04	NS
Smoking status (tobacco)	7% (4)	3% (2)	0.28–15.7	NS
Cannabis use	6% (3)	10% (6)	0.10–2.45	NS
Use of sperm-directed anti-oxidant formulation	17% (9)	34% (20)	1.25–19.8	*0.02*

P values in italics were statistically relevant

the only study specifically assessing a 3-h abstinence period on SDF levels included a small sample size of twelve men [22]. Therefore, this is the first study, to our knowledge, that has assessed improvements in SDF levels in all males presenting for fertility evaluation, including men with known male factor infertility. Relative to semen samples collected after a 3-day abstinence period, SDF levels in semen samples collected after 3 h of abstinence were significantly improved. Among the subset of patients with high levels of SDF (> 35%), 55% showed improvement into a normal range with the second ejaculation. Apart from the change in SDF levels in the second specimen, changes in sperm quality were not clinically significant. A small number (6%) of subjects had worse DNA fragmentation in the second specimen. However, all subjects where the DNA fragmentation got worse stayed within the normal range if they started out as normal. Similarity, none of the specimens that were designated as use in an IVF cycle would have required ICSI based on the semen analysis results of the second ejaculation 3 h later. Interestingly, men with higher initial sperm DNA fragmentation were more likely to improve by at least 30%, suggesting that significantly reduced abstinence periods may be of greatest benefit in men with higher levels of SDF. It should be noted that the greatest improvement in an individual seen in this study was 97% decreasing to 28% SDF.

Based on subgroup analysis, younger men and those taking a sperm-improvement vitamin supplement were more likely to have at least a 30% improvement in DNA-fragmentation on the second specimen. There was a slight but significant increased mean age of the men who displayed less than 30% improvement in SDF after a 3-h abstinence period (42.1 ± 8.1 vs. 40.3 ± 6.0 $p = 0.03$), which is in keeping with previously published studies suggesting that advanced paternal age is associated with accumulated damage to sperm DNA, increased sperm DNA fragmentation, and increased single gene mutations [25–27]. It is possible, therefore, that increased SDF associated with advanced paternal age is less likely to be adequately managed with shorter abstinence periods, although it would still be worth offering as a treatment option. Interestingly, cigarette smoking and cannabis use were not associated with increases in sperm DNA fragmentation in our population. Both cigarette smoking and cannabis use are well established as having deleterious effects on sperm quality, including increasing SDF [28–30]. Given the small percentage of subjects in our group who participated in these vices, our findings likely represent a type 2 error. Therefore, further research of a larger group of men is required to fully elucidate the impact of these habits, as well as advanced paternal age, on SDF and its improvement with a short abstinence period.

The results of this study suggest that high SDF levels can often be managed with a second ejaculation 3 h after the first. Second ejaculation provides an easy, non-invasive, and cost-free treatment for high SDF when compared to the currently available treatments of TESA and ANEXIN separation. Given that the rate of SDF improvements into the normal range after TESA and ANEXIN treatments are unknown, we hypothesized that a second, early ejaculation may be a mechanism to manage high sperm DNA fragmentation in ART, and further studies comparing results with ANEXIN separation and TESA are needed. Furthermore, TESA and ANEXIN separation techniques necessitate the use of ICSI to use the sperm collected. Although 7 out of 112 men in our study did not show an improvement in SDF levels with a shorter ejaculation window, 94% had improved SDF parameters, with some improving into a range appropriate for IUI treatment. A study previously conducted in 2010 revealed that abstinence times of less than 2 days when acquiring semen used in IUI treatments resulted in higher pregnancy rates [31]. Although these results were not directly attributed to improvements in sperm DNA fragmentation levels per se, they support the assertion that shorter abstinence periods can be useful in improving the quality of semen for IUI procedures. Therefore, in men with high sperm DNA fragmentation but otherwise normal semen parameters, a short window of ejaculation can likely be used to improve SDF levels in specimens that could normally be used for IUI treatment. Most importantly, in our experience, a second ejaculation is universally preferred by all male patients compared with the notion of needing a TESA. Currently, IUI is not an option with TESA or ANEXIN separations, representing the first solution for high SDF for IUI specimens. Importantly, a 3 h window of ejaculation is cost free and is not a barrier for production for most if not all of our patients. It should be noted that 7 subjects did not return for a second ejaculation, and although most attributed this to time constraints, erectile issues may have been present.

Compared with the high costs of TESA (thousands of dollars) and ANEXIN separation (hundreds of dollars), we suggest that a short window of ejaculation should be tried in all subjects with high DNA fragmentation, as a cost-free option to improving SDF levels. In specimens where DNA fragmentation levels only improve slightly after a 3 hour window of ejaculation, ANEXIN separation can be used as an additive technique. Although these additive techniques have shown promise in further improving sperm DNA fragmentation levels, further investigations are needed to determine if a short window of ejaculation plus ANEXIN is better than ANEXIN separation alone.

Currently, the literature assessing abstinence periods before producing semen samples used in ART treatments remains limited. Not only are there a small number of studies on this subject, but each study assesses different time periods, ranging from 3 to 48 h, and with different primary outcomes, including sperm concentrations reactive oxygen species and sperm DNA fragmentation, just to name a few [8, 21, 22, 31]. Therefore, additional studies should be performed to elucidate

the most ideal abstinence period on specific semen parameters, including sperm DNA fragmentation.

Despite the significant results of this study, it is not without its limitations. Primarily, the level of SDF was measured using the HALO sperm assay (sperm chromatin dispersion test), which estimates the level of DNA fragmentation indirectly by quantification of the amount of nuclear dispersion/halo after sperm lysis and acid denaturation to remove excess nuclear proteins [24]. The study could have been strengthened by using a direct measurement of SDF. Secondly, the study could have benefitted from a larger sample size to further elucidate factors which may influence SDF levels and predictors for SDF improvement. Since strict morphology was not measured in the second specimen, we cannot determine how the 3-h duration of abstinence affected this parameter. This was done due to cost constraints. The use of Viagra in six subjects may have affected the sperm DNA fragmentation, although it is too small a group to test and obtain legitimate results. Conversely, this study has much strength. This was a prospective, blinded cohort study, which significantly reduces the risk of selection and observer bias. It also used a simple intervention (second ejaculation) that is easily reproducible, thereby increasing external validity. Internal validity is enhanced based on the objective assessment of SDF using a readily available and validated assay (halo sperm test). The study was also adequately powered to detect significant differences between the two samples studied. Finally, this is the first study to date that assessed improvements in SDF levels after 3 h of abstinence in a population of men with male factor infertility as well as normal sperm parameters. This is also one of the first studies to develop a treatment for high DNA fragmentation which could also be used with intra-uterine insemination, although study of such is required.

Conclusion

High SDF can often be managed with a second ejaculation 3 h after the first in infertile couples. This represents a cost- and risk-free mechanism to manage elevated SDF fragmentation in sperm. All men with elevated SDF should be offered a repeat semen evaluation with 3 h of abstinence before being referred for TESA or ANEXIN separation. All men undergoing this protocol should also be prescribed a sperm-directed anti-oxidant formulation to increase their chances of SDF improvement.

Author contributions MHD, SLT, and GT conceived of the study. MHD and RK analyzed the data; AG, GT, and RK analyzed the DNA fragmentation or collected the data. MHD and GM wrote the article. GT, SLT, and RK edited the article. Both MHD and GM acted as the primary or first author for this study.

Funding This study was funded by a grant from the Montreal Reproductive and Regenerative Medicine Foundation.

Data availability Data is deposited.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethics approval IRB approval was obtained.

Consent to participate Patients consented to participate.

Consent for publication Patients consented to participate.

Code availability Not applicable.

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