The effect of culture media and seeding densities on the human malignant testicular germ cell line (NT2/D1) growth pattern

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Abstract

Testicular cancer is a common malignancy in young males, comprising 5% of urological cancers and 1% of all male cancers. The NT2/D1 cell line, a pluripotent embryonal carcinoma, is valuable in cancer and developmental biology research due to its differentiation potential into neurons and glial cells. Although Fetal Bovine Serum (FBS) is often used to promote cell growth, its limitations necessitate optimized culture conditions. Monitoring cell growth and health through methods such as cell counting, viability assays, growth curves, and microscopy is crucial for reliable research, particularly for evaluating specific cell characteristics before *in vitro* studies. The study compared the growth patterns of NT2/D1 cell lines under different culture conditions and seeding densities using trypan blue cell counting and MTT methods. The NT2/D1 cell lines cultured in heat-inactivated FBS (H.iFBS) and non-heat-inactivated FBS (Non-H.iFBS) exhibit distinct growth patterns, doubling times, and cell morphologies. Cell density and the condition of the culture medium are two key parameters influencing metabolic activities and enhancing variability. There is no preference between culturing cells in different FBS conditions yet it should be considered in experiment design. Thus, systematic reporting of all cell culture parameters is essential to facilitate better comparisons of *in vitro* and *in vivo* experimental data.

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Introduction

Testicular cancer is one of the most widespread neoplasms in men aged 20–40 and the most prevalent solid malignancy in young men. It constitutes 5% of urological malignancies and 1% of all cancers.[1] Testicular Germ Cell Tumor (TGCT) is a histologically heterogeneous disease derived from fetal gonocytes abnormally arrested in development, failing to mature into spermatogonia after birth. These arrested gonocytes acquire oncogenic genetic modifications during childhood and puberty, leading to germ-cell neoplasia *in situ* (GCNIS) and subsequently invasive TGCT in young adults.[2] Histologically, TGCTs are classified into seminoma and non-seminoma.[3]

The NT2/D1 cell line, derived from a 22-year-old male patient in 1984 by Andrews,[4] is a well-known human malignant testicular germ cell line. These pluripotent embryonal carcinoma cells are extensively used in studying embryonic development, differentiation, and diseases.[5,6] Their ability to differentiate into neurons and glial cells makes them valuable for neurobiological research[7] NT2/D1 cells have broad applications in cancer, developmental biology, neurobiology, and pharmacology research.[7–9]

Modern cell culture techniques allow precise control of the cellular environment, including nutrient concentrations critical for cell growth.[10] Fetal Bovine Serum (FBS), commonly used to support cell growth and adhesion, provides growth factors, amino acids, and vitamins but has limitations such as undefined composition, ethical concerns, and potential contamination.[11,12] In regenerative medicine and cell biology, identifying optimal culture con-

ditions that support efficient growth while maintaining inherent properties like self-renewal and multipotency remains a challenge.[13] This study focuses on characterizing human NT2/D1 cells under heat-modified and non-heat-modified FBS conditions.

Maintaining genetic stability over cell passages is crucial for reliable research outcomes. Regular validation of cell identity and characteristics ensures consistent experimental results.[14]

NT2/D1 cells offer insights into fundamental biological processes and serve as platforms for novel therapeutic developments.[8,9] Optimizing and monitoring their growth conditions ensures reliable, reproducible results, critical for experimental design, drug screening, and therapeutic development.[14,15]

Methods for assessing cell growth and health include cell counting, viability assays, metabolic activity assays, growth curve analysis, and microscopic observation.[16,17] These techniques provide quantitative and qualitative data on cell proliferation, viability, and health, enabling researchers to optimize culture conditions and protocols for specific needs.[16–18]

Cell viability and proliferation can be measured using methods like trypan blue dye exclusion and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assays. MTT, a colorimetric assay based on reducing MTT to formazan, is widely used for its simplicity, safety, and cost-effectiveness.[19,20]

Growth curve analysis plots cell counts or metabolic activity over time, revealing growth kinetics, exponential phases, doubling times, and saturation densities, which are critical for optimizing culture conditions and experimental designs.[21] Microscopic examination complements these methods by assessing morphology, adherence, and signs of stress, differentiation, or contamination.[14,22]

Before initiating *in vitro* cancer studies, evaluating cell-specific characteristics during the exponential growth phase is crucial.[23] However, factors like FBS conditions (heat-inactivated *vs.* non-heat-inactivated) and seeding density, which significantly affect cell growth patterns, are often overlooked. This study investigates how seeding density influences growth patterns and growth curve characteristics of NT2/D1 cells under two media conditions: heat-inactivated FBS (H.iFBS) and non-heat-inactivated FBS (Non.H.iFBS). Growth was analyzed using trypan blue dye exclusion with an inverted microscope and MTT cell viability assays.

Materials and Methods

Cell culture of human malignant testicular germ cell line NT2/D1

All cell culture was carried out using aseptic techniques and all cells were grown in T25 culture flasks (T25 Cell Culture Flasks - Vented Cap - Tissue Culture Treated, Biofargo,VA, USA). The human malignant testicular germ cell line NTERA-2 cl.D1 (NT2/D1), representing an embryonal carcinoma, was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). NT2/D1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose with L-glutamine (ATCC, Manassas, VA, USA) supplemented with either heat-inactivated or non-heat-inactivated 10% Fetal Bovine Serum (v/v) (Gibco BRL, Thermo Fisher Scientific, Waltham, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Life Technologies, Thermo Fisher Scientific, Waltham, USA) at 37°C, 5% CO_2 and 95% humidity. FBS was heat-inactivated after heating for 30 min in a water bath at 56°C.

In the incubated media, the adherent cells were removed mechanically. The cell flask was then monitored under the inverted light microscope (IM-3, OPTIKA, Ponteranica, BG, Italy) at 10× magnification. The cells were centrifuged at 5000 × g for 5 min at 25°C. The pellets were resuspended in 1 mL of fresh medium.

Hemocytometer cell counting

The amount of viable cells was determined using trypan blue exclusion assay a method described previously, as reported by Strober.[24] Briefly, the 80 % confluent NT2/D1 cells were cultured in 96 well plates at a density of 15,000 cells mL^{-1}(Con.1), 30,000 cells mL^{-1} (Con.2), 60,000 cells mL^{-1} (Con.3), and 120,000 cells mL^{-1} (Con.4) (100 µL cells suspension per well). Five plates (one plate per day each containing different densities) were prepared and the cells were seeded in triplicate. The reading of the initial number of cell seeding at 0 hours (day 0) was taken. One plate was counted daily over the five days (120 hours), and 10 µL of cell resuspension was mixed with 10 µL trypan blue dye. This mixture was pipetted onto an Improved Neubauer haemocytometer (Camlab, Cambridge, UK) (0.1 mm depth, 400 mm^{-2}). The cells were counted in the four (0.1 mm^3) corner squares, including those touching the left and bottom sides, using a light microscope at 10× magnification (Olympus CK40- 55 SLP, Tokyo, Japan). The cell density per mL was then calculated using the following formula; Cell density = cell number (mean from four fields) × 10^4 × dilution factor. This was done in triplicate wells in two different experiment sets.

MTT assay

The growth of NT2/D1 cells in different media conditions (H.iFBS and Non.HiFBS) was identified using MTT assay (Elabscience, Houston, Texas, USA). Briefly, the 80% confluent NT2/D1 cells were cultured in 96 well plates at a density of 15,000 cells mL^{-1}(Con.1), 30,000 cells mL^{-1} (Con.2), 60,000 cells mL^{-1} (Con.3), and 120,000 cells mL^{-1} (Con.4) (100 µL cells suspension per well). Five plates (one plate per day each containing different densites) were prepared and the cells were seeded in triplicate. The reading of the initial absorbance (Ab) of cell seeding at 0 hours (day 0) was taken Please, indicate the instrument (with model, provider, city and country). Over the five days (120 hours), one plate for each cell density were measured every day, a volume of 50 µL 1×MTT working solution (1:10 in DMEM) was added to each well including control wells (cultured media), and the plates were incubated at 37°C for 2h. After removing the media, 150 µL of dimethyl sulfoxide (DMSO) (Thermofisher, Waltham, USA) was added to each well to dissolve the formazan. The magnitude of the reduction reaction was determined by monitoring the absorbance of the solubilized formazan product at 570 nm using a plate reader (Agilent-BioTek Microplate Readers, Santa Clara, California, USA). Data is calculated as follows; *Ab of seeding well – Ab of control well (blank)*. This was done in triplicate wells in two different experiment sets.

Statistical analysis

Data analysis was carried out using GraphPad Prism software and statistically analyzed by Two-way ANOVA followed by Šídák's multiple comparisons. Growth curves and doubling time were analyzed by nonlinear regression (Exponential (Malthusian) growth). Results represent mean ± Standard Error of Mean (SEM) and a *p*-value less than 0.05 was considered significant.

Results

Human malignant testicular germ cell line (NT2/D1) growth curve determined by hemocytometer cell counting

As shown in Figure 1, the growth curve was determined by hemocytometer cell counting using trypan blue dye exclusion. Distinct growth curve patterns have been observed for NT2/D1 cells cultivated in both media conditions H.iFBS (Fig.1A) and Non.H.iFBS (Fig.1B). For NT2/D1 cells seeding in a low density of 15,000 cells mL^{-1} (Con.1), 30,000 cells mL^{-1} (Con.2) and 60,000 cells mL^{-1} (Con.3), the cells showed an increase in cell numbers over time reaching the maximum densities after 96 hours for H.iFBS and 72 hours for Non.H.iFBS. There were no variations in the growth curve pattern of the NT2/D1cells cultivated in Non.H.iFBS media between different seeding densities (Figure 1B).

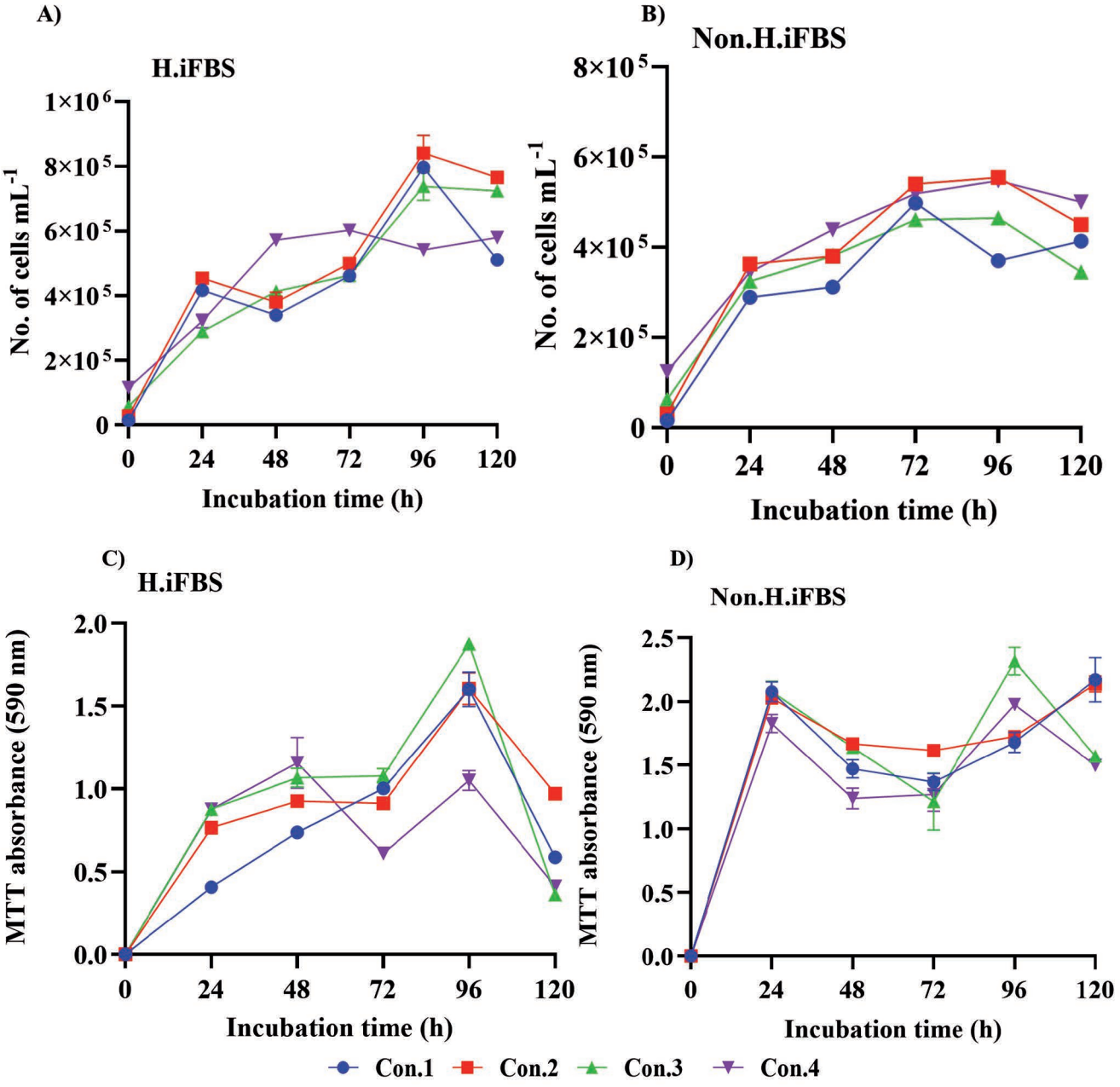

Figure 1. Human malignant testicular germ cell line (NT2/D1) growth curve determined by hemocytometer cell counting using trypan blue dye exclusion (A and B) or MTT assay (C and D) for two different media conditions. NT2/D1 cells were cultured in different seeding concentrations: 15,000 cells mL^{-1} (Con.1), 30,000 cells mL^{-1} (Con.2), 60,000 cells mL^{-1} (Con.3), and 120,000 cells mL^{-1} (Con.4), in two different media conditions over 120 hours (0 hours = initial reading as Control): A) Heat-inactivated FBS (H.iFBS) and B) Non-Heat-inactivated FBS (Non.H.iFBS) for hemocytometer cell counting using trypan blue dye exclusion and C) (H.iFBS) and D) (Non.H.iFBS) for MTT assay. The assay was performed twice in triplicate; Data are represented as mean ± Standard Error of Mean (SEM.).

Human malignant testicular germ cell line (NT2/D1) growth curve determined by MTT assay

The NT2/D1cells growth curve was also monitored using MTT assay. A similar growth pattern was obtained for NT2/D1cells cultivated in H.iFBS media (Figure 1C) to that determined by trypan blue dye exclusion (Figure 1A). These results indicated a good correlation between these two methods for cells cultivated in H.iFBS media. On the other hand, NT2/D1cells cultivated in Non.H.iFBS media showed a different growth pattern compared to that obtained from cell counting by trypan blue dye exclusion. The cells reached high optical density after 24 hours, followed by a slight decrease (Figure 1D). However, the two highest cell densities showed a maximum increase in cell viability after 96 hours of incubation, while the lowest cell densities showed a maximum increase in cell viability after 120 hours.

The statistical significance of data is better shown in Figures 2 and 3. The results showed significant differences in cell density between cells cultured in Non-H.iFBS and those cultured in H.iFBS media at different cell seeding densities. When using the cell counting method, we found that cells cultured in H.iFBS media had higher cell counts (Figure 2), while the MTT assay showed that cells in

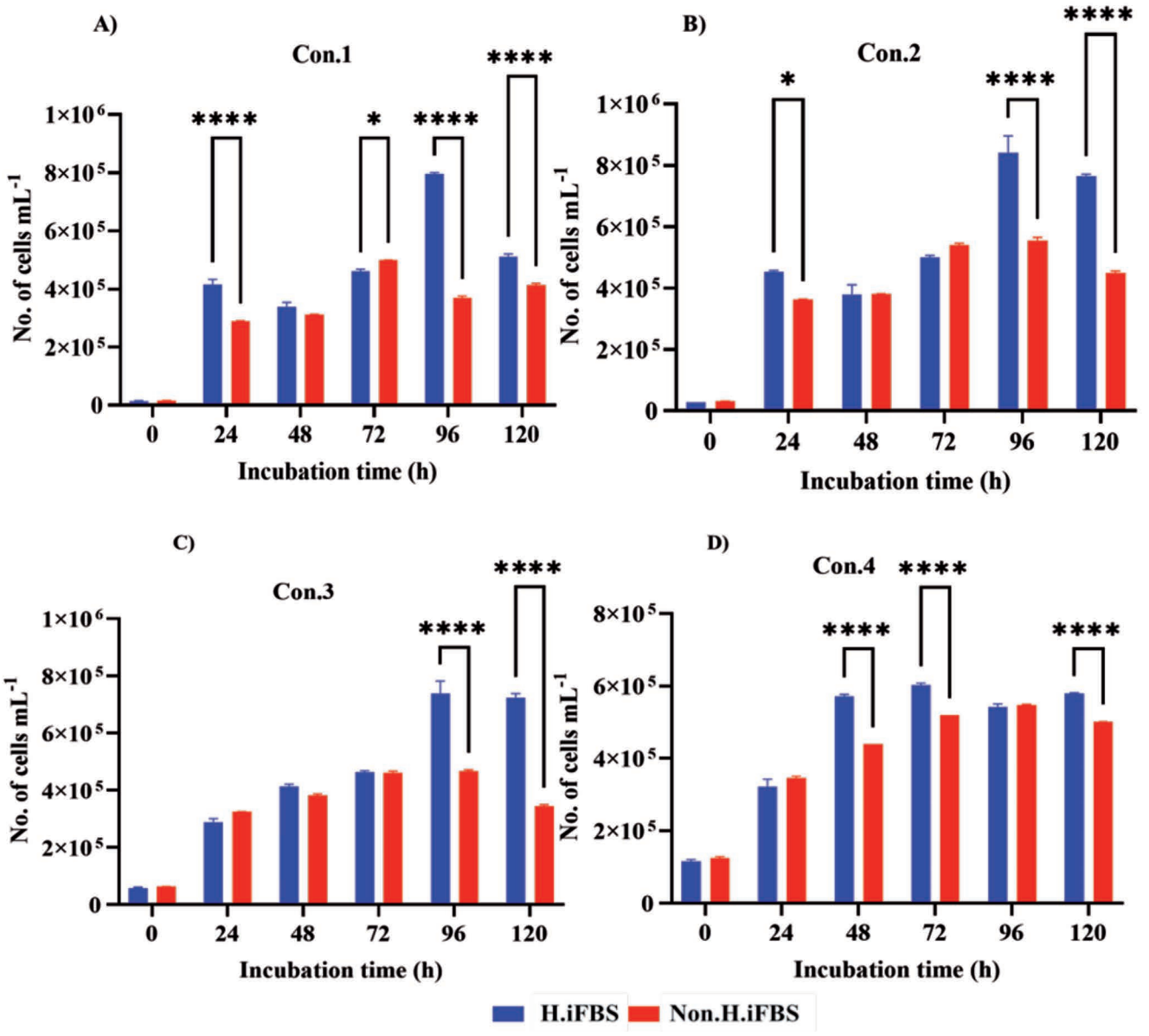

Figure 2. Effect of different media conditions on NT1/D2 cell density determined by hemocytometer cell counting. Analysis of NT2/D1 cell density monitored by hemocytometer cell counting over 120 hours for different seeding concentrations: A) 15,000 cells mL^{-1} (Con.1), B) 30,000 cells mL^{-1} (Con.2), C) 60,000 cells mL^{-1} (Con.3) and D) 120,000 cells mL^{-1} (Con.4), cultivated in media with Heat-inactivated FBS (H. iFBS) and Non-heat-inactivated FBS (Non.H.iFBS). The 0 hours represent the initial reading (Control). The assay was performed twice in triplicate; Data are represented as mean ± SEM. Data analysis was performed using Two-way ANOVA followed by Šídák's multiple comparisons. Statistical significance: *p<0.05, ****p<0.0001.

Non-H.iFBS media had higher optical density (Figure 3). Moreover, at seeding concentrations 15,000 cells mL^{-1}(Con.1), 30,000 cells mL^{-1} (Con.2), 60,000 cells mL^{-1} (Con.3) (Figure 2A, B, and C), the significant differences (n=2; ****p<0.0001) in cell count of H.iFBS media were noticeable after 96 and 120 hours compared to Non-H.iFBS media. While high significant differences (n=2; ****p<0.0001) were shown after 24 h at seeding concentrations 15,000 cells mL^{-1} (Con.1) (Figure 2A) and after 48 and 72 hours at 120,000 cells mL^{-1} (Con.4) (Figure 2D) . In contrast, all different seeding concentrations showed high significant differences (n=2; ****p<0.0001) in optical density of Non-H.iFBS media in comparison to H.iFBS media after 24 and 120 hours (Figure 3A, B, C and D) or after 48 hours (n=2; ***p<0.001 or **p<0.01) at seeding concentrations of 15,000 cells mL^{-1} (Con.1), 30,000 cells mL^{-1} (Con.2), 60,000 cells mL^{-1} (Con.3) (Figure 3A, B and C).

Doubling time of different NT2/D1 cell seeding densities determined in different cultured media

The NT2/D1cells Doubling Time (DT) was also calculated for both media conditions at different cell densities (Table 1) using

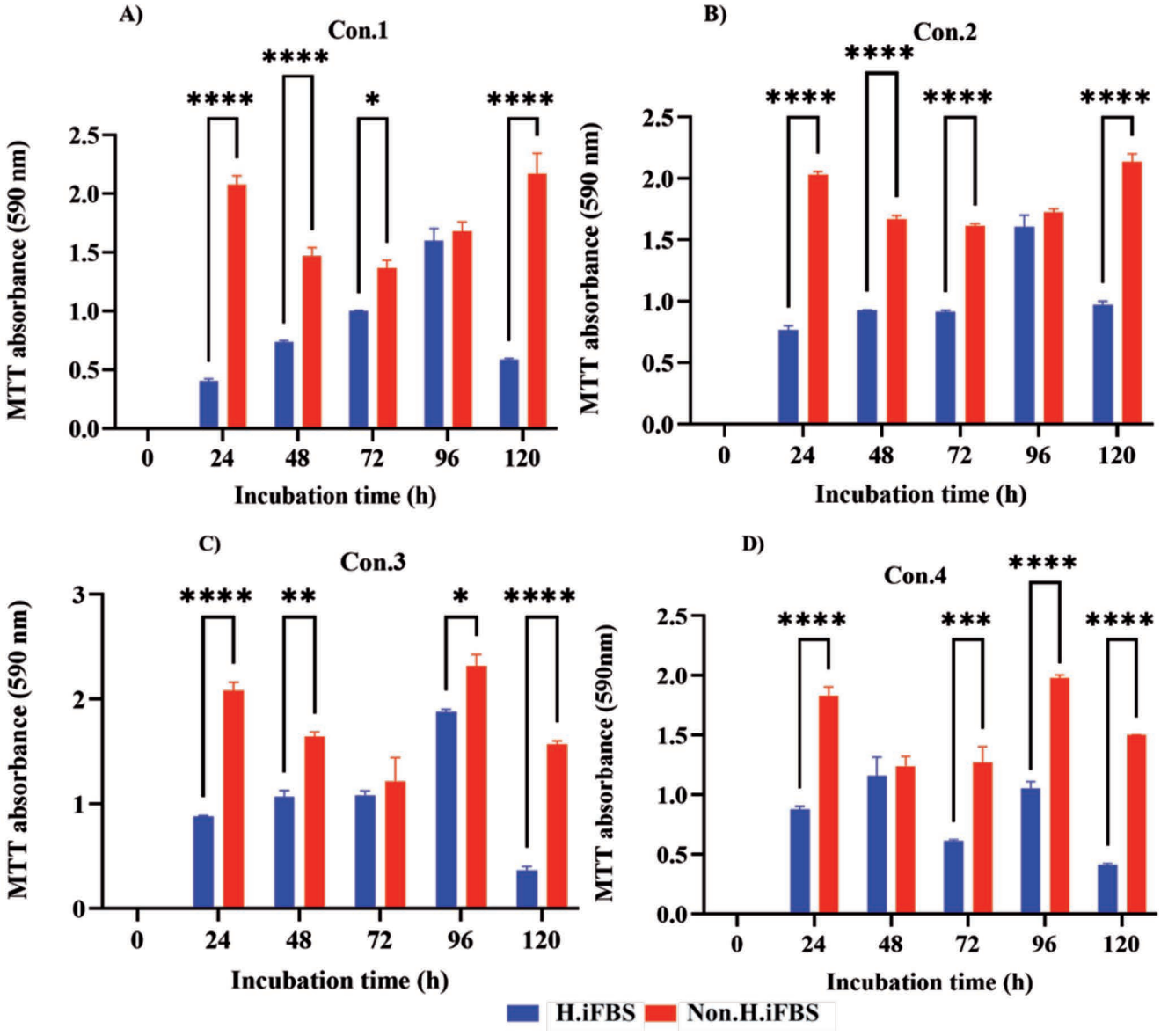

Figure 3. Effect of different media conditions on NT1/D2 cell density determined by MTT assay. Analysis of NT2/D1 cell density monitored by MTT assay over 120 hours for different seeding concentrations: A) 15,000 cells mL^{-1} (Con.1), B) 30,000 cells mL^{-1} (Con.2), C) 60,000 cells mL^{-1} (Con.3) and D) 120,000 cells mL^{-1} (Con.4), cultivated in media with Heat-inactivated FBS (H.iFBS) and Non-heat-inactivated FBS (Non.H.iFBS). The 0 hours represent the initial reading (Control). The assay was performed twice in triplicate. Data are represented as mean ± SEM. Data analysis was performed using Two-way ANOVA followed by Šídák's multiple comparisons. Statistical significance: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

the growth curve generated by cell counting and MTT methods. The cell doubling time was found to vary depending on the cell densities, incubation time, and media condition. Using the cell counting method, the DT for cells cultured in Non.H.iFBS was longer than those cultured in H.iFBS media. The DT ranged from 51 to 69 hours for cells cultured in Non.H.iFBS and 43 to 62 hours for cells cultured in H.iFBS media. Interestingly, the DT shows increasing when the seeding densities were too high. The DT determined by the MTT assay is higher than that observed using the cell counting method for both types of culturing media. This difference was significant at a seeding density of 60,000 cells mL^{-1} (Con.3) and 120,000 cells mL^{-1} (Con.4) for Non.H.iFBS media (n=2, ****p <0.0001), and at a seeding density of 15,000 cells mL^{-1} (Con.1) and 120,000 cells mL^{-1} (Con.4) for H.iFBS media (n=2,*p <0.05 and **p <0.01), respectively.

Cell growth characteristics of the NT2/D1cells line in different cultured media

To determine the cell growth characteristics of the NT2/D1cells line, the cells were monitored under the inverted light microscope for 120 hours. The highest seeding density (120,000 cells mL^{-1}) was chosen for both media conditions since the growth curve at this seeding density was the closest to the typical growth curve rather than the other cell seeding densities. As shown in Figure 4, the NT2/D1cells cultured in H.iFBS media exhibited a small rounded shape and rapidly attached to wells at the first 24 hours of culturing (Figure 4A, a). Many gaps were observed at this stage with fewer connected cells. Cells showed rapid division and proliferation and more defined morphology (epithelial-like cells) during the following 48-72 hours, forming a monolayer in which cells connect with fewer gaps (Figure 4A, b-c). After 96 hours, the cells reached 100 % confluence and the culture wells became crowded (Figure 4A, d). On day 5 (120 hours), the colour of the culture media changed to yellowish, due to a pH drop and some dead cells started floating (Figure 4A, e). In contrast to H.iFBS media, NT2/D1 cells cultured in Non.H.iFBS media displayed a small, rounded shape after 24 hours, with some cells floating and forming clusters (Figure 4B, a). Over the next 48 hours, the cells exhibited rapid division and proliferation, forming a crowded monolayer in the wells (Figure 4B, b). After 72 hours, the cells increased in size and exhibited a more defined shape, resembling epithelial-like cells. They became multinucleated and displayed less cytoplasm, with some cells appearing in groups that were separated by small gaps. Additionally, clusters of cells became detached (see Figure 4B, c). From 96 to 120 hours, the wells were once again fully populated with no gaps, although many clusters of cells detached (see Figure 4B, d-e).

Discussion

Optimizing and monitoring growth conditions of cancer cell lines is essential for obtaining reliable results and maintaining cell health. Careful consideration of initial cell numbers is crucial for successful differentiation and consistent experimentation. These practices support drug screening and therapeutic development while minimizing costs and resource wastage.[25,26] The NTERA-2 cL.D1 cell line, a human testicular carcinoma line, can differentiate into neuroectodermal lineages, making it valuable for embryogenesis research.[27–29] This study compared NTERA-2 cL.D1 growth under two conditions and seeding densities to enhance understanding of their behavior.

In the current study, we examined the cell growth pattern of the human malignant testicular germ cell line (NT2/D1) cultivated in two different media conditions (Heat-inactivated FBS and Non-heat-inactivated FBS) over 120 hours at different seeding concentrations. The growth curve was determined using trypan blue dye exclusion and distinct patterns were observed for both media conditions. For NT2/D1cells seeding at a low density, the cells showed an increase of cell numbers over time in which they reached the maximum densities after 96 hours for H.iFBS and 72 hours for Non.H.iFBS, suggesting that the cells were at log phase.[30] However, the seeding density at 120,000 cells mL^{-1} (Con.4) cultivated in H.iFBS showed the maximum increase in cell numbers after 48 hours and remained constant over the post-incubation period. This suggested that cells attained a stationary phase.[30,31] In this study we observed that at higher cell densities cells entered the death phase of the growth curve more quickly. This was accompanied by a faster change in media pH, indicated by a more rapid change in media color to yellowish. Previous reports suggested that when a certain number of cells is exceeded, a plateau level in Optical Density (OD) can occur.[32,33] This is thought to be caused by over-confluence resulting from the seeding of many cells. Consequently, there are changes in nutrient availability, cell viability, and metabolism.[33] NT2/D1 cells show consistent growth curve patterns in Non.H.iFBS media regardless seeding density (Figure 1B). This suggested that the initial seeding density in the case of Non.H.iFBS media may not have a significant impact on cell growth behavior. It has been reported that in the case of cancer cell lines, the seeding densities may not affect the lag time but can affect the doubling and saturated time.[34]

The NT2/D1cells growth curve was monitored using MTT assay. A good correlation was found between the MTT assay and trypan blue dye exclusion for cells cultivated in H.iFBS media. However, NT2/D1 cells cultivated in Non.H.iFBS media showed a different growth pattern compared to cell counting by trypan blue dye exclusion. According to a recent study, the time required for can-

Table 1. Doubling time (DT) of different NT2/D1 cell seeding densities in different cultured media determined by cell counting (CC) and MTT methods.

NT2/D1 cell seeding density	Heat-inactivated FBS (H.iFBS) DT/h		Non-heat-inactivated FBS (Non.H.iFBS) DT/h	
	CC	MTT	CC	MTT
Con.1=15,000 cell mL^{-1}	43.55**	37.68	51.28**	46.41
Con.2=30,000 cell mL^{-1}	42.56	44.97	54.84*	51.28
Con.3=60,000 cell mL^{-1}	44.89	44.64	64.98****	50.24
Con.4=120,000 cell mL^{-1}	62.01*	56.95	69.21****	50.18

*Asterisks represent the p-value of the comparison between NT2/D1 cell doubling time obtained from cell counting (CC) *vs.* MTT methods. Data analysis was performed using Two-way ANOVA followed by Šídák's multiple comparisons and represented as mean ± SEM of twice in triplicate. n=2, *p<0.05, **p<0.01, and ****p<0.0001. SEM values are missing

cer cells to adapt to their environment decreases as the initial cell density increases.[35] Furthermore, the density at which cells are initially seeded has an impact on the growth pattern of certain cancer cell lines, such as the human breast adenocarcinoma MCF-7 cell line, human ovary carcinoma cell line (A2780), and human melanoma cell line (A375). Lower seeding densities result in growth curves that follow a more predictable pattern, while higher seeding densities lead to different growth patterns.[34]

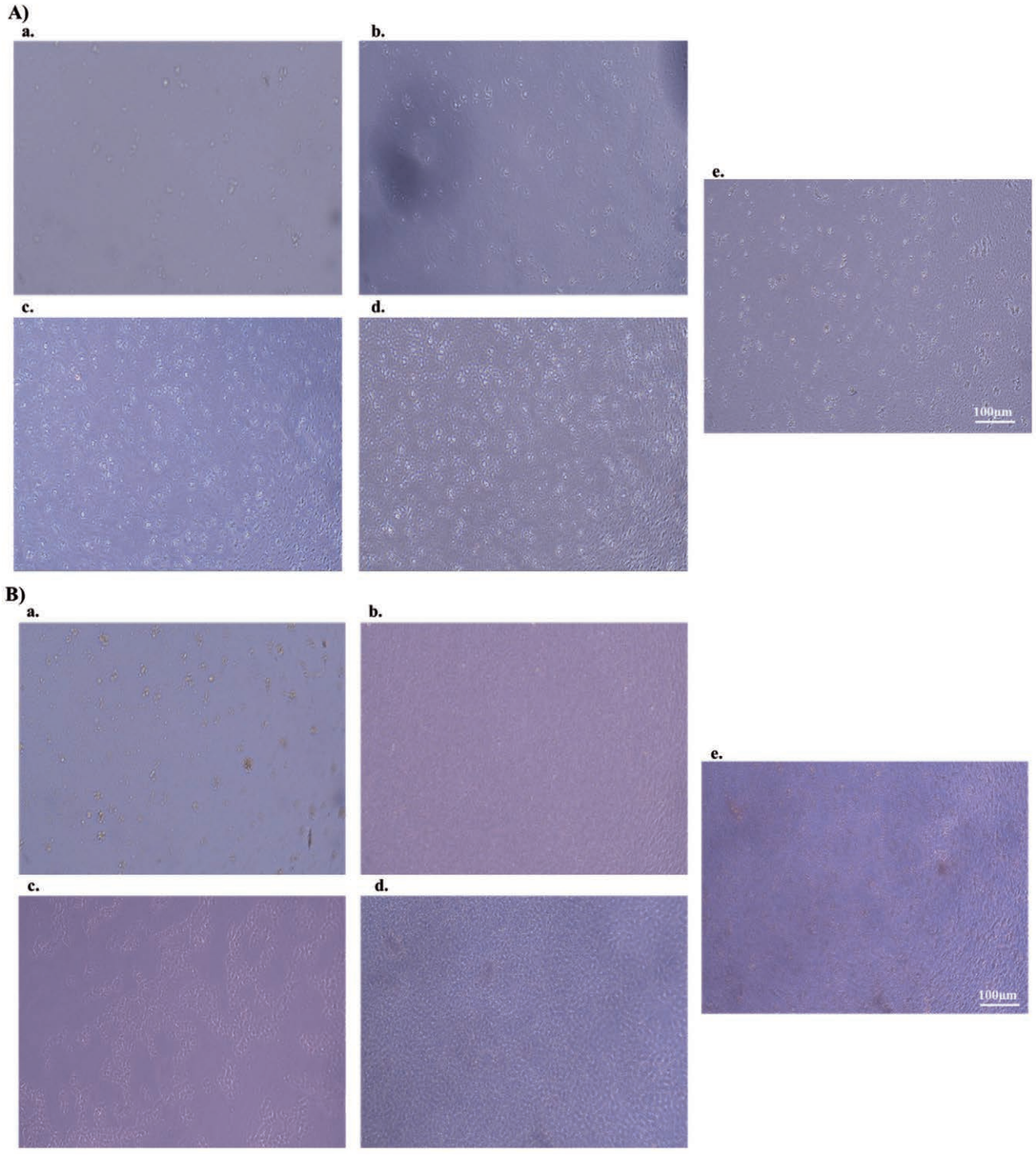

Figure 4. Photograph of the human malignant testicular germ cell line (NT2/D1) cultivated in Heat-inactivated FBS (H.iFBS) (A) or Non-heat-inactivated FBS (Non.H.iFBS) (B). The NT2/D1 cells were plated at a seeding density of 120,000 cells mL^{-1} and cultured in DMEM media of complete condition supplemented with 10% Heat-inactivated FBS (H.iFBS) or 10% Non-heat-inactivated FBS (Non.H.iFBS). Photographs of NT2/D1 cells during 120 hours (a. 24 h, b. 48 h, c. 72 h, d. 96 h, and e. 120 h) of culturing monitored under the inverted light microscope at 10× magnification. Scale bar 100 µm.

The study compared the impact of different media conditions on NT1/D2 cell density. The results showed significant differences in cell density between cells cultured in Non-H.iFBS and those cultured in H.iFBS media at different cell seeding densities. When using the cell counting method, we found that cells cultured in H.iFBS media had higher cell counts, while the MTT assay showed that cells in Non-H.iFBS media had higher optical density. Indeed, it has been reported that MTT assay may overestimate the cell viability in some cases and underestimate it in others and this depends on cell types, treatments concentration, and supplement medium.[33,36]

In the experiment, the doubling time of NT2/D1 cells was calculated for different cell densities and media conditions using cell counting and MTT methods and was seen to vary based on cell densities and media conditions. Cells cultured in Non.H.iFBS had longer doubling times than those in H.iFBS media. The doubling time increased with higher seeding densities. Additionally, the doubling time determined by the MTT assay was higher than that observed using the cell counting method for both media types. However, changes in OD levels do not always reflect changes in the percentage of viable cells. For example, doubling the number of cells does not necessarily double the OD levels.[10,37] Different studies have demonstrated that OD is not always directly proportional to cell number, and the relationship depends on cell type, number of cells, pH, and the formazan-solubilizing agent used.[37] Indeed, inconsistencies in growth patterns between the MTT assay and trypan blue exclusion assay are well-documented. These discrepancies stem from the different measurements each method evaluates and their sensitivities to various factors. The MTT assay measures metabolic activity by detecting the conversion of MTT to formazan by viable cells' mitochondrial enzymes, reflecting cellular metabolism that can remain high despite stress, indicating cells may appear viable without proliferating.[32,38] In contrast, the trypan blue exclusion assay counts live and dead cells based on membrane integrity, providing a direct measure of cell viability but not accounting for metabolic changes.[38] Moreover, variations in culture media, like using heat-inactivated versus non-heat-inactivated FBS, can affect cell proliferation and metabolic activity differently. Heat inactivation may reduce growth factors and nutrients, impacting proliferation more than metabolism. This can result in discrepancies where the MTT assay shows higher viability than the cell count.[32]

Seeding density affects cell behavior, including metabolic activity and nutrient use. High densities can cause contact inhibition, decreasing proliferation but maintaining metabolism, while low densities may slow metabolism due to limited cell interactions, impacting MTT results without changes in cell counts.[32,39] Furthermore, the MTT assay is affected by experimental conditions like assay duration and reagent concentrations, which may not directly correlate with cell number. It also struggles to differentiate between live and inactive cells.[39] Manual counting with trypan blue is susceptible to operator bias, especially at high cell densities where clumping occurs.[38]

In the experiment, NT2/D1 cells were monitored for 120 hours to determine their growth characteristics. The cells cultured in H.iFBS media rapidly attached to the well walls within 24 hours, showed rapid division and proliferation, and reached 100% confluence by 96 hours. On the other hand, cells cultured in Non.H.iFBS media formed clusters and detached from the wells after 72-120 hours. Although NT2/D1 cells in the different cultured conditions show the same general morphology of NT2/D1 cells that are characterized by little cytoplasm, prominent nucleoli, and growth in the form of clusters,[5,40] the size of cells and clusters and defined structure differed at the identical time point. The fact that we observed these differences in cell morphology and growth characteristics between cells cultured in H.iFBS media compared to cells cultured in Non.H.iFBS media is expected to be due to significant amounts of Extracellular Vesicles (EVs) present on FBS, which can impact *in vitro* studies.[41] EVs present in FBS are naturally produced by the bovine cells in the developing fetus or during processing.[42] These vesicles are part of the normal cellular communication system, carrying proteins, lipids, RNA, and other bioactive molecules.[43] Bovine EVs present a challenge when using FBS as a cell culture media supplement, as they may contaminate cell-derived EV samples and influence the growth and phenotype of cultured cells.[44] For this reason, many studies typically heat-inactivate the FBS at 56°C for 30 to 60 minutes before use to avoid the risk of contamination.

Moreover, numerous studies have shown that the heat inactivation of FBS can hinder cell growth, phenotype, and genotype.[13,45] This can explain the findings of this study. However, it was recommended by the American Tissue Culture Collection (ATCC) that the NT2/D1cells should be cultured in Non.H.iFBS media as this increases cell proliferation by maintaining growth factors. Moreover, the floating cells present in the culture media are not necessarily dead cells, but they are rather viable cells that provide growth factors and other components greatly needed for the whole population to complete their growth and decrease the time between subcultures.[46,47] Thus, it is recommended not to discard media at passage time after 2-3 days yet collect cells by centrifuge and return them to the cultured flask. This should be considered especially if experiments require prolonged incubation. This type of cell is also recommended to be cultured in high densities and trypsin-Ethylenediaminetetraacetic Acid (EDTA) should be avoided as this may damage the cells.[47,48]

The study involved comparing the growth curves of NT2/D1 cell lines under different culture conditions and seeding densities using trypan blue cell counting and MTT methods. The MTT assay was the most efficient, with less experimental error. However, it has a significant drawback compared to trypan blue cell counting assay, as it does not allow for the observation of cellular morphology, especially in the context of the cell growth curve, and its results can be affected by cultured media conditions. In addition, it is important to note that there is no favor in the culture of NT2/D1 cells when using heat-inactivated FBS (H.iFBS) or non-heat-inactivated FBS (Non-H.iFBS). However, it should be considered that NT2/D1 cells exhibit distinct growth patterns and doubling times when cultured in different FBS conditions. This is crucial when planning experiments. For instance, stable cell growth patterns and doubling times are essential for obtaining accurate drug dose-response and reliable IC50 in drug screening.[49] This stability may be achieved with FBS (H.iFBS) due to its lower levels of active complement proteins, resulting in reduced interaction with quantitation assays.[12,13] Moreover, for experiments focusing on maximizing cell growth or proliferation rates, we recommend using Non-H.iFBS, as our results show that it supports slightly enhanced proliferation compared to H.iFBS. The preservation of heat-sensitive growth factors and other nutrients in Non-H.iFBS likely contributes to this effect, making it ideal for experiments requiring rapid cell expansion or large-scale cultures.[13,32] For experiments requiring stable cell morphology or induction of differentiation, H.iFBS is preferred. Our findings indicate that H.iFBS conditions result in a more consistent cell morphology with less variability. This is likely due to the reduced activity of heat-sensitive growth factors that could otherwise disrupt cellular homeostasis, aligning with reports that H.iFBS facilitates controlled differentiation and minimizes interference from extraneous growth factors.[14] While both types are useful for the general culturing of NT2/D1 cells, the choice should align with the experimental objectives. For example,

drug screening studies may benefit from Non-H.iFBS to ensure high metabolic activity, while differentiation or morphological studies may require the consistency provided by H.iFBS.

Nevertheless, our results indicated a strong correlation between the growth curves obtained using MTT and trypan blue dye exclusion with heat-inactivated FBS (H.iFBS). Thus, culturing NT2/D1 cells in heat-inactivated FBS (H.iFBS) or non-heat-inactivated FBS (Non-H.iFBS) primarily depends on experimental design and should be determined at the beginning of experiments to ensure reliable research findings. Thus, we propose the following guidelines for selecting culture conditions depending on the experimental objectives: for drug cytotoxicity or efficacy testing, Non-H.iFBS is recommended. Non-H.iFBS retains heat-sensitive growth factors and nutrients that promote robust metabolic activity, which is crucial for accurate drug-response profiling using metabolic assays like MTT. Higher seeding densities (e.g., 1.2×10^5 cells mL^{-1}) are advised to ensure uniform cell attachment and reproducibility across replicates.[32,38] For differentiation studies, H.iFBS is preferred, as heat inactivation minimizes serum-derived growth factors that may interfere with differentiation signals. This aligns with findings that H.iFBS supports controlled differentiation in human pluripotent stem cells and other cell lines by reducing external influences.[14] Moderate seeding densities (e.g., 3.0×10^4 cells mL^{-1} and 6.0×10^4 cells mL^{-1}) are recommended to allow sufficient room for cellular morphological changes and clear observation of phenotypic transitions. For routine culture and expansion of NT2/D1 cells, both serum types are viable options, depending on resource availability. However, Non-H.iFBS is preferred for maximizing proliferation rates, as our findings show enhanced growth under these conditions. This recommendation aligns with reports emphasizing the role of Non-H.iFBS in maintaining robust growth in various human cell lines.[13]

Conclusions

This study highlights the significant impact of media conditions and seeding densities on the growth patterns, doubling times, and metabolic activity of NT2/D1 cells. Results demonstrated that Non-H.iFBS promotes enhanced cell proliferation and higher metabolic activity, making it suitable for experiments requiring rapid expansion or drug screening. In contrast, H.iFBS fosters consistent morphology and supports controlled differentiation, which is advantageous for studies on cellular development or morphology.

The findings also underline the importance of aligning cultural conditions with experimental objectives. Non-H.iFBS is ideal for assays relying on metabolic activity, while H.iFBS is better suited for differentiation and morphological consistency. Despite some discrepancies between MTT and trypan blue assays, both methods provided complementary insights into cell growth behavior. Researchers should carefully consider media conditions and seeding densities during experimental planning to ensure reproducibility and accurate results, tailoring protocols to the specific needs of their study.

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